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Veur Pap en Mam

Abbreviations

BCE	bovine microvascular endothelial cells
BPI	bactericidal/permeability-increasing protein (BP55, bactericidal protein of 55 kD)
BsAb	bispecific antibody
BSA	bovine serum albumin
CAM	chick chorioallantoic membrane
DNA	deoxyribonucleic acid
EAM	endothelial adhesion molecule
ECGS	endothelial cell growth supplement
EC(s)	endothelial cell(s)
EM	extracellular matrix
ELISA	enzyme linked immunosorbant assay
FCS	fetal calf serum
aFGF, FGF-1	acidic fibroblast growth factor
bFGF, FGF-2	basic fibroblast growth factor
FGF(s)	fibroblast growth factor(s)
HS	heat inactivated human pooled serum
HUVEC	human umbilical vein derived endothelial cells
IFN	interferon
IL-8	interleukin 8
LPS	lipopolysaccaride
MMP(s)	matrix metalloproteinase(s)
MRI	magnetic resonance imaging
MVEC	human microvascular endothelial cells
PA	plasminogen activators
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PF4	platelet factor 4
PI	propidium iodide
PKC	protein kinase C
SPARC	secreted protein acidic and rich in cysteine
TGF	transforming growth factor
Tie-2	angiopoietin receptor
TNF	tumor necrosis factor
TSP	thrombospondin
TUNEL	terminal transferase-mediated UTP nick-end labelling
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
ZVAD.fmk	Z-Val-Ala-Asp-fluoromethylketone

Contents

Chapter 1

General introduction
Angiogenesis inhibition, research and application

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Introduction

In solid tumor growth various stages can be discriminated. After multiple genetic changes, cellular growth progresses via hyperplasia and dysplasia towards an in situ tumor. Only after the so called angiogenic switch, an as of yet ill defined set of molecular changes leading to the formation of new blood vessels, a tumor progresses towards a mass extending more than several millimeters in diameter[1]. By recruitment of new blood vessels, the tumor provides itself with a continuous supply of nutrients and paves the way for metastasis formation. Many tumors diagnosed in the clinic have a size beyond 0.5 cm in diameter and are considered pro-angiogenic. The architecture of a solid tumor is such, that numerous layers of tumor cells are fed by one blood vessel. Consequently, several layers of tumor cells are not well accessible for drug targeting preparations. In contrast, endothelial cells (ECs) lining the tumor vasculature are quite easily accessible for macromolecular preparations. Therefore, they are an interesting target for the delivery of pharmacologically active agents. Furthermore, their pivotal role in maintenance of tumor cell survival and growth also makes them an interesting target. Tumor growth does not expand more than several millimeters in diameter in the absence of new blood vessel formation. Hence, growing tumors such as the majority observed in the clinic are characterized by the formation of new blood vessels from existing ones, a process called angiogenesis. ECs are important executioner cells in the angiogenic cascade. Interference with this pro-angiogenic character by inhibiting pro-angiogenic signal transduction in ECs may provide important ways to interfere with tumor growth. The majority of research on inhibiting the function of tumor vasculature focuses at the development of drugs that may have an explicit action on the ECs in the tumor. Based on their mechanism(s) of action and the fact that angiogenesis is a normal physiological process, these drugs in theory can also interfere with normal EC function in the body.

At present, most target epitopes capable of discriminating tumor endothelium from normal endothelium are molecules that are expressed during angiogenesis. Therefore, in this chapter a summary of up to date knowledge on tumor growth related angiogenic endothelial processes in angiogenesis will be given. Furthermore, the currently known angiogenesis inhibitors and preclinical experience with these will be discussed. Finally, the aim of research and outline of this thesis will be given.

Functions of vascular endothelial cells in the body

The vasculature is one of the main organs in the body, extending more than 900 m^2 and playing a major role in maintaining the body's integrity in various ways. Blood vessels consist of ECs, which are directly in contact with the blood, and subendothelially located pericytes, smooth muscle cells, fibroblasts, basement

membrane, and extracellular matrix (EM). Depending on the location in the body and the organ microenvironment, the cellular constituents, basement membrane and EM differ in phenotype, composition, and function[2]. The ECs form a monolayer in every blood vessel in the circulation. They are actively involved in several regulatory processes in the body. Besides being metabolically active and selectively permeable for small solutes and peptides/proteins, ECs are actively involved in regulating haemostasis[3]. Furthermore, they are able to recruit cells of the immune system to specific sites of e.g. infection or inflammation by virtue of the regulated expression of cell adhesion molecules and production of cytokines and chemokines[4]. Lastly, ECs are actively involved in vascular remodeling in for example ovulation, wound healing, tumor growth and diabetic retinopathy[4;5].

Molecular control of tumor growth related angiogenesis

In adult life, physiologic stimuli during wound healing and during the reproductive cycle in women lead to controlled angiogenesis. However, pathologic conditions such as tumor growth, rheumatoid arthritis, and diabetic retinopathy are characterized by abundant angiogenesis.

Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions. In tumor growth, this active vascular remodeling is reflected by enhanced tumor endothelial cell (EC) proliferation to up to 20 - 2000 times faster than in normal endothelium in the adult[6]. In all types of angiogenesis, either under physiologic or pathologic conditions, EC activation is followed by matrix degradation, cellular migration, proliferation, and ultimately neovasculature maturation (Figure 1.1).

Role of growth factors VEGF and bFGF

More than twenty cytokines from various sources have now been described to be involved in the processes taking place during angiogenesis[7]. Vascular EC growth factor (VEGF) and basic Fibroblast Growth Factor (FGF-2 or bFGF) are two growth factors whose roles in angiogenesis have been most extensively studied to date. VEGF (also known as VEGF-A) isoforms VEGF-121, 145, 165, 183, 189 and 205 are a result of alternative splicing from a single VEGF gene located on chromosome 6[8]. The isoforms differ in their molecular weight and biological properties. The larger forms of VEGF differ from VEGF 121 by the presence of a heparin-binding domain, which is encoded by the exon-7. VEGF is abundantly produced by hypoxic tumor cells, macrophages and other cells of the immune system[9]. It induces vasodilatation via endothelial nitric oxide production and increases EC permeability[10]. This allows plasma proteins to enter the tissue to form a fibrin rich provisional network[11]. VEGF also induces the expression of proteases and receptors important in cellular invasion and tissue remodeling, activates cellular proliferation and prevents EC apoptosis[12;13]. The two VEGF specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/flk-1), are expressed on vascular endothelium, and to a lesser extent on monocytes/ macrophages and certain tumor cell types. Interaction of VEGF with VEGFR-2 is a

critical requirement to induce the full spectrum of VEGF biologic responses. In addition to the two VEGF receptors, VEGF165 has been found to bind neuropilin-1, which is also expressed on ECs[14]. A recent study using genetic deletion methods has determined that neuropilin-1 is important for embryonic vessel formation[15].

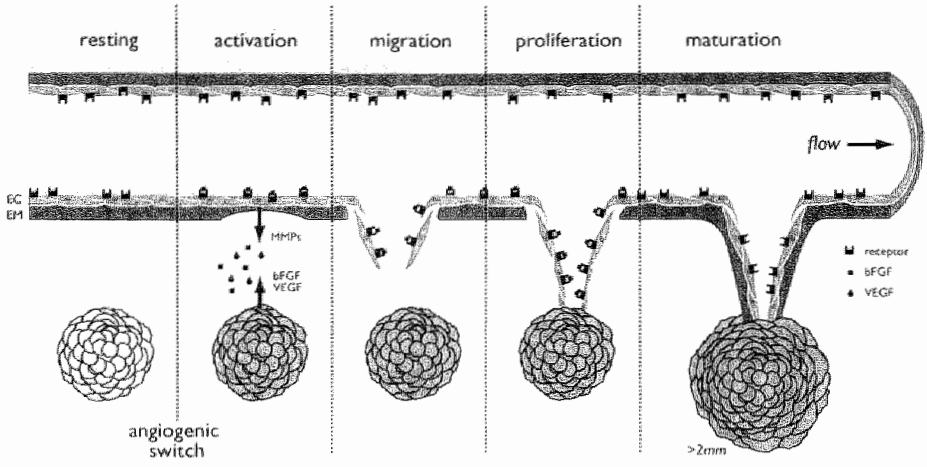

Figure 1.1 *The angiogenic cascade.*
A tumour can only grow to a diameter of approximately 2 mm without new blood vessels. After the so called angiogenic switch a tumour starts producing angiogenic factors, such as bFGF and VEGF which diffuse in the direction of a nearby capillary and there the factors bind to receptors present on the endothelial cells (EC) lining the vessel. The endothelial cells are thereby activated and produce matrix metalloproteinases (MMPs) that degrade the extracellular matrix (EM), which facilitates the migration of the endothelial cells in the direction of the tumour. The endothelial cells are also activated to proliferate and ultimately polarize to form a lumen. Maturation occurs via the formation of extracellular matrix and attraction of pericytes.

ECs exploit various proteases such as matrix metalloproteinases to penetrate into new areas of the body by degrading the basement membrane. Furthermore, plasminogen activators (PA) urokinase-PA and tissue type-PA convert the ubiquitous plasma protein plasminogen to plasmin. Plasmin is believed to be the most important protease for the mobilization of bFGF from the EM pool. bFGF induces EC motility, proliferation and proteinase activity, and modulates integrin levels[16;17]. The cellular effects of Fibroblast growth factor (FGFs) are mediated via specific binding to high-affinity tyrosine kinase receptors, of which four have been identified[16]. In addition, low affinity FGF receptors exist, which consist of polysaccharide components of heparan sulfate proteoglycans on cell surfaces and EM. Binding to the latter receptors present in the EM has been proposed as a mechanism to stabilize and protect FGF from inactivation. Heparan sulfate on cell surfaces, on the other hand, play a more active role in displacing EM-bound bFGF and its subsequent presentation to the high affinity signal transducing receptors[18].

Of note is the observation that angiogenesis seems exquisitely sensitive to small changes in factors such as VEGF and bFGF. This may have important therapeutic implications in treatment of angiogenesis driven disorders[19;20].

Role of integrins

Integrins are transmembrane proteins composed of an α and β subunit in over twenty different αβ heterodimeric combinations. They bind to EM proteins or cell surface ligands through short peptide sequences and are also implied in angiogenesis control. Combinations of different integrins on (endothelial) cell surfaces allow cells to recognize and respond to a variety of different EM proteins[21]. They are able to transduce signals from within the cells to the outside as well as from the outside into the cell[22]. Integrin mediated cell adhesion impacts two key aspects of growth regulation. First, it can influence the activity of the basal cell cycle machinery consisting of cyclin dependent kinase complexes. Second, integrins play a vital role in anchorage dependent cell death or anoikis[23;24]. For example, integrin $\alpha_v\beta_3$ mediates EC adhesion to vitronectin, fibrinogen, laminin, collagen, von Willebrand Factor or osteopontin through their exposed tripeptide Arg-Gly-Asp (RGD) moiety[25]. Since $\alpha_v\beta_3$ is minimally expressed on normal resting endothelium, but significantly upregulated on tumor and other activated endothelium, it is believed to play a critical role in the process of angiogenesis. Both peptide and antibody inhibitors of $\alpha_v\beta_3$ induced EC apoptosis, suggesting a role for this integrin in EC survival during angiogenesis[26]. Another α_v integrin associated with angiogenesis is $\alpha_v\beta_5$. Whereas bFGF or tumor necrosis factor α (TNFα) induced $\alpha_v\beta_3$ dependent angiogenesis *in vivo*, VEGF or transforming growth factor β (TGF-β) initiated an angiogenesis pathway merely dependent on $\alpha_v\beta_5$[27].

Role of the extracellular matrix

Components of the EM play an important role in the regulation of EC morphology and function. Thrombospondin (TSP), for example, can affect EC proliferation negatively as well as positively, depending on the endothelial microenvironment. Furthermore, through binding to and activation of TGF-β and affecting protease activity, TSP may be able to influence cell growth, migration and differentiation[28]. Laminin also plays a role in cell attachment, growth promotion, protease secretion and interactions with other EM components. It can bind to cell surface binding proteins including integrins, which leads to integrin signaling[29]. SPARC (Secreted Protein Acidic and Rich in Cysteine), also known as BM40 or osteonectin, is a protein of which the expression is elevated under stress conditions. Transient expression of SPARC during EC injury and cellular activation indicate a role in tissue repair, remodeling and angiogenesis[30]. Exogenously added SPARC or SPARC derived peptides were able to modify EC behavior via the induction of proteases and inhibitors of plasmin generation[31;32].

Role of subendothelially present support cells

EC interaction with EM and mesenchymal cells is a prerequisite to form a stable vasculature. Therefore, after EC proliferation and maturation, and the formation of endothelial tube structures, surrounding vessel layers composed of mural cells (pericytes in small vessels and smooth muscle cells in large vessels) need to be recruited. ECs may accomplish this via the synthesis and secretion of platelet derived growth factor (PDGF), a mitogen and chemoattractant for a variety of mesenchymal cells. Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be a cell-cell contact dependent process. Upon EC - mural cell contact, a latent form of TGF-β, produced by both endothelium and mural cells, is activated in a plasmin-mediated process. Activated TGF-β can induce changes in myofibroblasts and pericytes that may contribute to the formation of a mature vessel, EM production and maintenance of growth control[33]. The coinciding investment of growing capillaries by pericytes with the deposition of basement membrane and cessation of vessel growth during wound healing also indicates vessel growth regulation by pericytes[34]. The aFGF receptor is also implicated in EC differentiation leading to vascular tube formation. Besides inducing plasminogen activator, and EC proliferation and migration, aFGF receptor signaling resulted in endothelial tube formation in collagen[35].

Assays and models

Specificity of blood vessel targeting to eradicate solid tumors depends on altered physiological processed in the tumor vasculature relative to normal vasculature in healthy tissues. It is therefore required to fundamentally investigate the vascular biology and cell biological events in vessel formation. A number of different assay systems and angiogenesis models can be used for the research underlying development of vascular targeting techniques for the treatment of cancer.

Endothelial cell sources

Pivotal for research on vessel formation, angiogenesis and angiogenic EC targeting is the availability of viable ECs, the cells that line the inside of blood vessels and govern the majority of angiogenic processes. ECs can be obtained from various tissues, purified and cultured *in vitro*. One should realize that under most culture conditions, ECs are never in quiescent state and can therefore limitedly serve as a model for resting vasculature. The best available source of human ECs is the large vein in the umbilical cord. Because this vein is not branched, it can be filled with collagenase to enzymatically detach the ECs from the vessel wall. ECs in culture start cell cycle and form a confluent monolayer on the tissue culture plastic. It should always be kept in mind that cultured ECs are never in quiescent state and can therefore limitedly serve as a model for resting vasculature. For

reason of simple isolation most laboratories make use of these human umbilical vein ECs (HUVECs). The major drawback of these cells is their macrovascular origin, which makes them less suitable for studies on angiogenesis, a microvascular process. Although more laborious, human microvascular ECs can be isolated from other organs such as foreskin or adipose-tissue. ECs in culture need a constant supply of growth factors such as FGFs and VEGF in order to continue cell cycle. In most cultures the addition of serum to the culture medium is sufficient to maintain a low level of EC proliferation. Cells cultured this way can be subcultured at a split ratio of 1:3 for 4-5 passages without significant loss of growth potential. Addition of low concentrations of recombinant or purified growth factors such as bFGF will increase the number of passages to 10-12. The limited subculture possibilities make repeated isolations necessary introducing significant variation of the EC source. To circumvent these drawbacks it is possible to immortalize ECs with viral oncogenes such as simian virus-40 large T antigen. Transfection of ECs with a DNA construct containing the gene for this molecule can result in cell lines that can be subcultured over 60 times. Examples of such cell lines are HMEC-1[36], EA.hy926[37], ECL4n[38], or EvL[39]. The major problem with these immortalized ECs is that they are genetically not stable, resulting in loss of phenotypic and functional characteristics and functional resemblance with their *in vivo* counterparts. Furthermore, many EC lines can be cultured on plastic tissue culture material, without being dependent on integrin signaling via EM molecules for growth and survival. Careful interpretation of results obtained with these cell lines is therefore necessary.

ECs from other species are also available. Popular are capillary ECs from bovine origin, because these cells are very sensitive to treatment with angiogenesis inhibitors such as angiostatin and endostatin (Griffioen et al, unpublished results). It should, however, be kept in mind that mechanisms of action can be different in the different species.

Functional assays with endothelial cells

Angiogenesis is a multi-step process, depending on activation, migration, proliferation and differentiation of ECs, and all these stages in the cascade can be studied independently.

Cell growth assays

Proliferation of ECs can be studied in multiple assays. Since the cell cycle potential of ECs is low, with cell doubling times of sometimes over 35 hours, counting of cells is a time consuming and non-sensitive way of cell growth assessment. A much better way to determine proliferation is assessment of [^{3}H]-thymidine incorporation[40;41]. Other assays to study proliferation of ECs, and other mechanisms of cell activation that do not involve S-phase of cell cycle, are based on colorimetric systems that measure mitochondrial activity (CCK-8, XTT or MTT). Alternatively, proliferation of ECs can be analyzed by DNA profiling, for example by flow cytometric analysis of cells in G_0/G_1 phase (2n DNA), cells in

G_2/M phase (4n DNA) and cells in S phase (2<n<4) after permeabilization and staining with propidium iodide. Also, proliferation can be quantified by determination of cell cycle dependent expression of molecules such as proliferating cell nuclear antigen (PCNA[42] or Ki-67[43]).

The net result of cell growth is also determined by the level of cell death. Therefore, detection of cell death is a commonly used approach to average cell growth; e.g. apoptosis induction can be studied most easily by detection of subdiploid cells or analysis of DNA degradation profiles on the flow cytometer after DNA extraction and propidium iodide staining. Apoptosis can also be quantified by enumeration of cells with fragmented nuclei by staining with e.g. propidium iodide or acridyl orange. Nick end labeling in terminal transferase-mediated UTP nick-end labeling (TUNEL)-analysis by immune-histochemistry or flow cytometry is currently regarded as specific detection of apoptosis. In order to get a qualitative indication of apoptosis induction analysis of DNA ladder formation in an agarose gel can be applied. Measurement of annexin-V binding to cells[44], which is based on changes in membrane asymmetry, records early signs of apoptosis.

Adhesion and migration assays

In order to form new vasculature ECs, when angiogenically activated, migrate through the EM. Two sequential steps in the angiogenesis cascade are fundamental to this process. The first step is the production of matrix metalloproteinases (MMPs), which dissolve the EM to facilitate migration of ECs. Measurement of MMP1 (collagenase-1), MMP2 (gelatinase A), MMP3 (stromelysin-1) and MMP9 (gelatinase B), which are proteinases correlated to angiogenesis and tumor growth, can be measured in ELISA, histochemistry or in Western blots. In the subsequent step ECs use their adhesion molecule make-up, which is intricately regulated by a plethora of (vasoactive) molecules involved in angiogenesis, to start the actual migration towards the angiogenic stimulus. Expression of adhesion molecules involved in matrix binding such as $\alpha_v\beta_3$-, $\alpha_v\beta_5$- and β_1-containing integrins and functional studies investigating binding to matrix components is an important part of angiogenesis research[45]. For the development of carrier molecules for drug targeting strategies aimed at tumor vasculature expressed $\alpha_v\beta_3$ integrin, these adhesion assays are suitable systems to test carrier specificity[46]. More functional studies address the process of migration using so-called Boyden chambers[47]. In this assay the migratory capacity of ECs after activation with a chemoattractant or an angiogenic is studied. Velocity of migration and percentage of cells that are capable of migrating through an EM coated membrane into another compartment are analyzed. The wound assay[48] is another way to measure EC migration. This assay system is based on the wounding of a confluent monolayer of ECs and the subsequent repair or closing of the wound by migration of ECs. This assay can be performed on different matrix components. The above-described technologies can be used to pinpoint the action spectrum of angiogenic or angiostatic agents to specific steps in the angiogenesis cascade. Application of these systems revealed

that IFNα and angiostatin inhibit migration whereas e.g. endostatin and platelet factor 4 function primarily as inhibitors of EC proliferation.

In vitro angiogenesis assays

The advantage of the above-described assays is the control over the few parameters present. A more complex experimental setup that studies consecutive steps in the angiogenic cascade is represented by three-dimensional *in vitro* models. In these models, ECs are cultured on top of a matrix gel (i.e. collagen[49], fibrin[50], or matrigel[51]) and are induced to form sprouts into the matrix by stimulation with either growth factors, tumor biopsies or tumor cell lines grown as spheroids. The criterion in all these assays is that a lumen is formed in these sprouts and that not merely migration and cell rearrangement is observed. In another assay ECs grow on gelatin coated beads which then are embedded in a matrix, after which the ECs are induced to form sprouts into the matrix[52]. The sprouting can be quantified either by measuring the distance over which vessels were formed or by computer based measuring of the total vessel length.

Other methods, reflecting the *in vivo* situation more closely, include assays based on ECs sprouting from freshly isolated tissues embedded in matrix gels, which include the rat aortic ring[53] and human placenta tissue[55]. This procedure is not applicable for all tissues as e.g. tumor biopsies often produce too much proteases digesting the matrix and thereby preventing EC sprouting[49]. A recently published novel way of measurement of angiogenesis *in vitro*, which is even more close to the *in vivo* situation, is the use of embryoid bodies[54]. *In vitro* cultured mouse blastocyst cells[55] recapitulate several steps of murine embryogenesis, including vasculogenesis and angiogenesis[56]. There is a complete blood vessel development in these embryoid bodies[57] making this system suitable for the study of angiogenesis modulators.

The advantages of *in vitro* assays are 1) the ability to control the assay variables, 2) the possibility of dissecting the complete process in its separate components, 3) the cellular and molecular events can be more carefully monitored and 4) the costs and time consumption are less than for *in vivo* assays. The disadvantages of *in vitro* assays include that the cells, reagents and conditions used in the different laboratories are not standardized and that the *in vitro* effects seen do not have to be the same *in vivo*, which has been demonstrated for e.g. TNFα, which *in vitro* inhibits angiogenesis, but *in vivo* induces angiogenesis[5]. Especially in the light of the influence of cells of the immune system on angiogenesis, extrapolation of data from *in vitro* to *in vivo* need to be carefully addressed.

In vivo assays for studying targeting of angiogenic blood vessels

It is well recognized that *in vitro* angiogenesis assays clearly have advantages, however, the major drawback of all these assays is that they require the ECs to be removed from their natural microenvironment, which may alter their physiologic properties. To study angiogenesis *in vivo*, the most frequently used assay systems exploit chicken chorioallantoic membrane (CAM)[27;58], the corneal pocket[59],

transparent chamber preparations such as the dorsal skin fold chamber[60;61], the cheek pouch window[62] and the polymer matrix implants[63;64].

The CAM assay is based on the developmental angiogenesis occurring in the CAM during chick embryo development. The developing vasculature can easily be observed and regulators of angiogenesis can be tested in this model by either intravessel injection or by addition of soluble compounds within a silicone ring placed onto the membrane or by release from a methylcellulose or alginate pellet. This assay is relatively inexpensive and is not considered an animal experiment by law, which can be an advantage in countries with strict animal experimentation legislation.

The corneal pocket assay and the window preparations are designed to measure vessel formation after addition of stimulators. These assays can e.g. be used for the study of angiogenic potential of human tumors. Also these models are very suitable for preclinical testing of angiogenesis inhibitors.

Implantation of polymer matrices that contain angiogenic factors requires quantification of the amount of vessel ingrowth. This can either be analyzed by immune-histochemistry or hemoglobin/red blood cell count. These analyses normally do not allow analysis of the vascularization in time, as they require sacrifice of animals. Application in a dorsal skin fold chamber circumvents this experimental problem as it gives the opportunity to compute vessel formation at various time points during the experiment.

In vivo assays have, however, also a number of disadvantages; e.g. the compound pharmacokinetics, necessary for proper interpretation of results, are often not known and the host might respond nonspecifically to the implantation. For a review on *in vivo* angiogenesis models and its potentials and problems, the reader is referred to[65].

Angiogenesis inhibition

Tumor induced angiogenesis can be modulated at different levels, for example by inhibition of growth factor or growth factor receptor signaling or EC growth, by directly effecting the ECs at the level of adhesion or migration, by inhibition of EC migration and by interference with the MMPs. The most investigated angiogenesis inhibitors are depicted in Table 1.1.

Interference with endothelial cell growth

ECs in normal vasculature are quiescent and divide approximately once in 6 months[91] and about 0.01% of ECs is in S-phase at any given time. In contrast, at areas of active angiogenesis, e.g. in tumor growth, wound healing and reproductive tissues undergoing tissue remodeling, ECs divide rapidly. Increased proliferation in these areas is accompanied by over-expression of growth factor receptors involved in angiogenesis. Some of the well-characterized receptor systems involved in angiogenic response are VEGF receptors, angiopoietin receptor (Tie-2), FGF receptor and endoglin.

VEGFR-1 and VEGFR-2, are over-expressed on tumor vasculature, while being present at low density in the surrounding normal tissues[92]. The upregulation of VEGFR expression is mediated by hypoxia and autocrine stimulation.

Angiogenesis can be inhibited by blockade of the over-expressed growth factors or their receptors[93]. This can be achieved by treatment with humanized blocking antibodies to these factors, antibodies to the receptors or soluble receptors to antagonize receptor binding, dominant negative growth factor variants, or VEGF antisence constructs. Also, receptor signaling can be blocked as has been described for SU5416, which specifically inhibits VEGFR-2 phosphorylation[94].

Table 1.1 Angiogenesis inhibitors and their function

Inhibitor	Mechanism of action
Angiostatin[66;67]	Inducer of EC apoptosis
	Inhibitor of EC proliferation
Endostatin[68;69]	Inducer of EC apoptosis
	Inhibitor of EC proliferation
Thrombospondin-1[70;71]	Inducer of EC apoptosis
	Inhibitor of EC proliferation
	Inhibitor of EC migration
16 kDa prolactin fragment[72]	Inhibitor of EC proliferation
	Inhibitor of bFGF induced angiogenesis
Interferon-α[73]	Inhibitor of EC migration
	Inhibitor of EC proliferation
	Inhibitor of bFGF induced angiogenesis
IP-10, platelet factor 4[74;75]	Inhibitor of EC proliferation
	Inhibitor of bFGF and IL-8 induced migration
Interleukin-4[76]	Inhibitor of EC migration
Bactericidal/permeability-increasing protein (BPI)[77]	Inhibitor of EC proliferation
	Inducer of EC apoptosis
	Inhibitor bFGF induced migration
Anginex[78;79]	Inhibitor of EC proliferation
	Inducer of EC apoptosis
	Inhibitor of bFGF induced migration
Batimastat/marimastat[80;81]	Inhibition of MMP activity
Minocycline[82]	Collagenase inhibitor
Vitaxin/LM609[83]	Blocks EC adhesion
	Inducer of EC apoptosis
Thalidomide[84]	Not known
SU5416/SU6668[85;86]	Blocks VEGF signal transduction
Suramin[87]	Binds to growth factors
	Inhibitor EC migration and proliferation
Combretastatin A4[88]	Binds tubulin
	Blocks EC proliferation
TNP-470[89;90]	Blocks cell cycle
	Inhibitor of EC proliferation

Since growth factor receptors undergo endocytosis upon ligand binding, VEGF was initially studied for its ability to deliver toxin polypeptides. VEGF165 was chemically linked to a truncated form of diphtheria toxin (DT385) by disulfide bond. VEGF-toxin conjugate was found to be very effective in inhibiting EC

proliferation *in vitro* and experimental angiogenesis *in vivo*[95]. Cytotoxicity to ECs was specific and dependent on VEGFR expression. Free toxin molecules did not show any effect on EC viability. VEGF-toxin conjugate treatment of tumor bearing mice showed selective vascular damage in tumor tissue and inhibited tumor growth[96]. The differential effect of VEGFR targeting on tumor vasculature is possibly contributed by three factors; 1) over-expression of VEGFR in tumor vessels leading to increased homing of VEGF-toxin conjugate. 2) proliferation dependent sensitivity to the effector moiety in the conjugate. 3) polarized distribution of VEGFR.

Chemical conjugates used in the earlier studies were prepared by random derivatization of VEGF with bifunctional reagents. Such methods often result in a heterogeneous mixture of different stoichiometry. To avoid batch-to-batch variations and to obtain structurally well-defined toxin conjugates, the coding region of VEGF and toxin polypeptides were fused at the DNA level. Unlike the chemical conjugates (dimeric VEGF linked to toxin moiety), genetically fused proteins were expressed as monomeric VEGF fused to toxin moiety. Interestingly, the monomeric constructs were found to be biologically active and inhibited tumor growth in mice[97]. Based on these observations, it was concluded that it is possible to re-engineer VEGF-toxin conjugates to optimize their anti-angiogenic effect.

Other growth factor receptors over-expressed in tumor vessels are FGF receptor and Tie-2 receptor. There have been few studies thus far to evaluate the relative merits of targeting via FGF to inhibit tumor blood vessels. Davol et al prepared an EC specific cytotoxic conjugate[98] by chemically linking a plant derived ribosomal inhibitory protein, saporin to FGF. FGF-saporin conjugate inhibited proliferation of ECs very effectively in *in vitro* studies. Recently, a fusion protein containing placenta growth factor and saporin was found to exert anti-angiogenic activity[99]. Since proliferating ECs show upregulation of a variety of proliferation associated cell markers such as transferrin receptors, it is also possible to target transferrin receptor to inhibit angiogenesis or directly attack tumor blood flow. Monoclonal antibodies reactive to human transferrin receptor have been used to prepare cytotoxic conjugates containing recombinant ricin A chain. These constructs were found to inhibit human corneal ECs in a proliferation dependent manner[100]. Targeting transferrin receptor will affect both proliferating endothelium and tumor cells and may therefore be useful at least for local application (e.g. excessive proliferation of blood vessels following eye injury) if not for systemic therapy. This application has not been widely studied in tumor vasculature targeting strategies. In contrast, extensive experience has been obtained in the brain targeting area.

Besides attacking growth factors or growth factor receptors there are angiogenesis inhibitors known that directly block cell cycle progression of ECs. AGM-1470/TNP-470, for example, is an analog of the fungus-derived antibiotic fumagilin[101;102] that prevents ECs to enter G_1 phase of the cell cycle[90]. Also endogenous inhibitors of EC growth have been described. Examples of these are

thrombospondin-1[103], platelet factor 4 (PF4)[104], interferon inducible protein-10[74], angiostatin[105] and endostatin[69].

Inhibition of adhesion or migration of endothelial cells

Interaction between cell surface anchored integrins and EM components constitute an additional pathway necessary for angiogenesis. In fact, recent studies have identified two cytokine mediated, integrin dependent angiogenic pathways. One of these pathways is associated with $\alpha_v\beta_3$ integrin, which selectively influences bFGF mediated angiogenic signals[27]. A second non-overlapping pathway is represented by crosstalk between $\alpha_v\beta_5$ integrin and PKC dependent growth factor mediated signaling which is VEGF or TNFα dependent[106]. Tumor angiogenesis can therefore be inhibited by blocking the interaction between integrins and RGD-motif containing EM proteins. Furthermore, the integrins present on tumor endothelium can serve as target epitopes via which toxic compounds can be delivered in the tumor ECs.

Erkki Ruoslahti and colleagues[107] developed a novel targeting strategy by using polypeptides capable of delivering cytotoxic drugs to integrins. An *in vivo* selection of phage display libraries identified peptides that specifically home to components of tumor blood vessels. Ruoslahti's research group identified two major classes of peptides, one containing the RGD motif and the other containing a NGR motif. These polypeptides were then chemically linked to the anti-cancer drug doxorubicin. Treatment of breast carcinoma bearing mice with the conjugated doxorubicin caused vascular damage in the tumors and a strong anti-tumor effect at a 10-40 times lower concentration than free doxorubicin, while liver and heart toxicity were less compared to those with free doxorubicin[107]. Whether this effect was caused by the selective delivery of the chemotherapeutic drug to the tumor ECs and/or tumor cells, direct caspase-3 activation[108], or a combination has not been studied yet. Their results illustrate the potential use of targeting therapeutic or toxic agents to integrins expressed on the vasculature of tumor cells as an effective means of cancer treatment.

The usefulness of the different methods of angiogenesis inhibition were for most substances demonstrated in various animal models, but still have to prove their effectiveness in clinical studies. For this purpose, clinically useful target epitopes on tumor endothelium need to be identified.

Some 40 different anti-angiogenic agents are currently in clinical trials. Examples of these are marimastat, AG3340, neovastat, TNP-470, thalidomide, CAI, SU5416, anti-VEGF antibody, and angiostatin (e.g. see NCI homepage). It needs to be established whether these drugs can be considered candidates for application in future drug targeting strategies. Targeting drugs to tumor ECs in order to inhibit tumor blood flow is an attractive approach for the treatment of solid tumors. Potentially, one of the advantages of such a treatment may be the lack of the occurrence of drug resistance, as tumor ECs are considered genetically stable. As tumor therapy aims at complete eradication of tumor cells, a combination of tumor vasculature directed strategies (anti-angiogenic drugs as such or targeted

drugs discussed here), and tumor cell directed chemotherapeutics and/or immunotherapy is at present believed to be the most optimal approach for future clinical application.

Aim of the presented research

Cancer is a pathological disorder of which the incidence increases with age. Following the second world war the economic and social climate improved considerably causing the so called baby boom of the 1950's and 60's. As the 21st century begins these baby boomers are approaching old age. It has been calculated that in the first two decades of this century the number of cancer patients will increase by 25% with many of them being over 70 years old. Another reason for the increase in cancer patients is that the economic growth and western lifestyle are more hazardous, for example smoking and high fat intake increase respectively the prevalence of lung cancer and colon cancer. In the 20th century research focused mainly on radiotherapy, surgery and chemotherapy. These modalities have been able to cure approximately 50% of cancer patients. During the 1990's, however, there appeared to be little prospect to further enhance these treatments. A rapidly increasing understanding of (molecular) cancer biology has changed this perspective. New therapies are being developed at specific targets within the cancer cell with drugs being rationally designed rather than being sought by serendipity as had been largely the case in the 20th century. Good examples of this new generation of agents include Glivec, a c-kit inhibitor that has proven extraordinarily effective in chronic myeloid leukemia and Iressa an epidermal growth factor tyrosine kinase inhibitor being developed for solid tumors such as lung cancer[109]. Similar approaches are being developed in the field of angiogenesis and this is the focus of this thesis. In the 1970's Dr. Folkman hypothesized that tumors required blood vessels to grow beyond a certain size and metastasize[110]. Later he showed that tumor blood vessels had a different phenotype from normal vessels, which could be specifically targeted for therapeutic purposes. Most of the early anti-angiogenic agents were developed from naturally occurring molecules, which were shown to be over expressed in tumor endothelium.

We used a new technology for the development of a new angiogenesis inhibitor. Most endogenous angiogenesis inhibitors contain numerous hydrophobic and cationic residues. Another feature of many angiogenesis inhibitors is the presence of an anti-parallel β-sheet, which is present in for example endostatin, PF4, tumor necrosis factor (TNF) and BPI. A library of peptides was designed in a combinatorial approach employing basic folding principles and incorporation of amino acid sequences of β-sheet containing angiogenesis inhibitors[79;111;112]. The 3D-structure of these anti-angiogenic proteins were used to design the βpep library of peptides. By the use of *in vitro* anti-angiogenesis assays one of the peptides in the library, βpep-25 that we called anginex, was identified as being the most effective in proliferation inhibition and apoptosis induction.

The approach used to identify new angiogenesis inhibitors as described shows a lot of potential. It uses a rational design, which can be adapted, and not 'on a wild hunch' testing of substances in angiogenesis assays. By replacing specific amino acids the surface active residues can be determined, which also plays a role in designing improved peptides or mimetics. In a next step these mimetic molecules can be made that are designed based on the peptide sequences. The development of mimetics could lead to compounds with a more favorable pharmacokinetic profile as well as being orally bioavailable. It also is possible to do mechanistical studies and studies to determine the receptor on ECs. Since the peptides are made synthetically the good medical practice traject new medicines have to go through is not as strict as it is for existing or recombinant compounds.

Outline of the thesis

Using the traditional approach for angiogenesis inhibitor finding in our laboratories, BPI was identified as possible angiogenesis inhibitor. Chapter 2 describes the identification of BPI as angiogenesis inhibitor. BPI until recently was only known as anti-bacterial and research was mainly focussed of research on its role as a treatment of sepsis. In chapter 3 the development of anginex (βpep-25) as an angiogenesis inhibitor is described. This is a 33 amino acid de novo designed peptide, whose design was based on the β-sheet structure of BPI, PF4 and interleukin-8. Chapter 4 describes the anti-angiogenic and anti-tumor efficacy of anginex in mice. In chapter 5 the mechanism of anginex in the inhibition of angiogenesis is outlined. Chapter 6 discusses the possible commonality between angiogenesis inhibitors and bactericidal substances. Chapter 7 is a general discussion and finally a summary is given in chapter 8.

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Chapter 2

Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells

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Abstract

Bactericidal/permeability-increasing protein (BPI) has been known for some time to function in killing bacteria and neutralizing the effects of bacterial endotoxin lipopolysaccharide. In the present study, BPI is found to be a novel endogenous inhibitor of angiogenesis. Within the sub-μM range, BPI shows a concentration dependent inhibition of endothelial cell (EC) proliferation which is mediated by cell detachment and subsequent induction of apoptosis. As measured by flow cytometric analysis of the percentage of subdiploid cells, apoptosis induction was half-maximal at about 250 nM BPI. Apoptosis was confirmed by quantification of cells with nuclear fragmentation. Apoptosis was found to be EC specific. In an *in vitro* collagen gel-based angiogenesis assay, BPI at 1.8 μM inhibited tube formation by 81% after only 24 hours. Evidence for *in vivo* inhibition of angiogenesis was obtained using the chorioallantoic membrane (CAM) assay in which BPI was seen to be significantly effective at concentrations as low as 180 nM. This newly discovered function of BPI might provide a possible therapeutic modality for the treatment of various pathologic disorders which are dependent on angiogenesis.

Introduction

The 55kD bactericidal protein produced by human neutrophils is most well known as the bactericidal/permeability increasing factor (BPI)[1;2], but it is also known as the cationic anti-bacterial protein of 57 kD (CAP57)[3] and as the bactericidal protein of 55 kD molecular mass (BP55)[4;5]. BPI is most bactericidal against Gram-negative bacteria[1] and has been shown to neutralize both the pyrogenicity of bacterial lipopolysaccharide (LPS) in rabbits and the ability of LPS to initiate the coagulation pathway of the *Limulus* amoebocyte lysate or to cause tumor necrosis factor (TNF) release from human monocytes[6]. BPI functions by binding to the lipid A moiety of LPS[7], while neutralizing the ability of this endotoxin to upregulate the expression of complement receptors on neutrophils and to cause a systemic inflammatory response during bacterial infection. Lipid A consists of a phosphorylated diglucosamine moiety attached to two fatty acyl chains. Since BPI is cationic and is known to bind the polysulfated gylcosaminoglycan heparin[8] it may function by effectively interacting with the anionic phosphate groups on LPS. Relatedly, many proteins isolated from blood are cationic and bind heparin. One of these, platelet factor 4 (PF4), is a potent anti-coagulant, perhaps the strongest binder of heparin, anti-angiogenic and is also known to be bactericidal[9;10].

Up until now, aside from its anti-bacterial effects, no other activities for BPI have been reported. Given chemical similarities between BPI and PF4 and the fact that they share at least some biological activities, it was postulated that they may display other functional similarities. Probably the most clinically important activity of PF4 is its ability to inhibit angiogenesis[11;12]. Angiogenesis, or the outgrowth of capillaries from existing vasculature, occurs during physiological processes such as embryogenesis, the menstrual cycle and wound healing, but it is also involved in numerous pathologic disorders requiring vascular outgrowth such as cancer, arthritis and atherosclerosis. A major interest in angiogenesis has developed in the field of oncology since it has been recognized that tumors, which are dependent on angiogenesis for outgrowth and metastasis, can be treated by attacking their blood supply[13;14].

Here it is reported that BPI, like PF4, is indeed an effective inhibitor of angiogenesis. We found that BPI inhibits endothelial cell (EC) growth at low doses leading to the inhibition of angiogenesis both *in vitro* and *in vivo*. The commonality between anti-angiogenic and anti-bacterial proteins and the role of angiogenesis during inflammation are discussed. The fact that BPI is now found to be an endogenous inhibitor of angiogenesis, makes it an important pharmacologic candidate for future anti-angiogenic therapy in the clinic.

Materials and methods

Purification of BPI

Venous blood was obtained in the form of leukocyte concentrates from the Red Cross, St. Paul, MN. Erythrocytes were lysed in a two step process involving ammonium chloride and hypotonic shock. Cytoplasmic granules were obtained by centrifugation as described previously[4], except that PMNL were lysed by nitrogen cavitation. BPI was then purified by column chromatography as previously described[5]. In the final step the sample was applied to a 1 × 180 cm molecular sieving column of Toyopearl HW55S (TosoHaas, Philadelphia, PA) which had been equilibrated with 0.05 M glycine buffer, pH 2.5 containing 0.5 M NaCl. Protein concentration was determined according to Hartree. Purity was confirmed by visualization of an SDS polyacrylamide gel following electrophoresis of 1 µg of purified BP55 protein and silver staining of the gel, also as previously described[15].

Cell culture

Human umbilical vein derived endothelial cells (HUVEC) were isolated form the vein of human umbilical cords and routinely cultured in RPMI-1640 (Life Technologies, Breda, The Netherlands) supplemented with 20% heat inactivated human pooled serum (HS, provided by the University Hospital Maastricht), 2 mM L-glutamin (Life Technologies, Breda, the Netherlands), 50 ng/ml streptomycin and 50 U/ml penicillin (ICN Biomedicals) in a 0.2% gelatin coated 75 cm^2 tissue culture flask at 37°C at 5% CO_2. HUVEC used for experiments were cultured without addition of extra growth factors and used for experiments between passage 2 and 4. Human microvascular endothelium (MVEC) was isolated from foreskins and cultured in the same medium.

Bovine microvascular endothelial cells (BCE) were kindly provided by Dr. M. Furie (State University of New York, Stony Brook, USA) and were cultured on gelatin coated flasks in MEM- (Life Technologies, Breda, The Netherlands) supplemented with 15% fetal calf serum (FCS, Boehringer Ingelheim Biowhittaker, Verviers, Belgium) and 50 U/ml penicillin and 50 ng/ml streptomycin.

Proliferation assay

HUVEC were seeded in a 96 wells culture plate coated with 1 mg/ml fibronectin (2 hours at 20°C) at a concentration of 3,000 cells per well in a volume of 50 µl. The cells were allowed to adhere for 3 hours at 37°C at 5% CO_2 and subsequently 50 µl of culture medium with 20 ng/ml basic Fibroblast Growth Factor (bFGF), with or without BPI was added. The cells were cultured for 72 hours. During the last 6 hours of the assay, the culture was pulsed with 0.5 µCi

[methyl-^{3}H]-thymidine (Amersham Life Science)/well. Activity was measured using liquid scintillation. Measurements were done in triplicate.

Detachment of cells was measured in HUVEC cultured as described above and with or without the control proteins bovine serum albumin (BSA) and platelet factor 4. Cultures were analyzed under an inverted microscope. In a microscopic field of 40 μm^2 adhered and detached cells were counted to give an indication of the amount of dying cells in time. At day 3 detached cells were collected, adherent cells were trypsinized and all cells were stained by mixing equal volumes of cell suspension and trypan blue solution (2 mg/ml, Serva, Germany). The percentage of dead cells was calculated after counting under an inverted microscope. Analysis of significance in differences was performed with the Mann-Whitney U test.

Apoptosis assay

HUVEC were seeded at a concentration of 3,000 cells per well in a 1 mg/ml fibronectin coated 96 wells culture plate. The cells were cultured during 72 hours either in normal culture medium, in the presence of BPI, PF4, BSA at different concentrations, or under serum deprivation. In some experiments cells were cultured with or without 5 I.U. heparin (Leo Pharmaceutical Products b.v., Weesp, Netherlands). After 72 hours of culture the cells were harvested with 0.125% trypsin. Part of the cells were used to make cytospin preparations on glass slides for determination of cell morphology after hematoxillin/eosin staining. The remaining cells were centrifuged at 1500 rpm for 5 minutes, washed once with PBS, and subsequently fixed in 70% ethanol at -20°C for 2 hours. The cells were pelleted at 1500 rpm and resuspended in DNA extraction buffer (90 volumes 0.05 M $Na_2HPO_4.2H_2O$, 10 volumes of 0.025 M Citric Acid and 1 volume of 10% Triton-X100 (in distilled water, pH7.4)) and incubated at 37°C for 20 minutes. After this incubation period propidium iodide (PI) was added at a concentration of 20 µg/ml and the DNA profile of the HUVEC was analyzed on the flow cytometer (FACS-calibur, Becton Dickinson). In some experiments the caspase inhibitor ZVAD.fmk (Alexis Biochemicals) was used (100 µM) to inhibit apoptosis[16]. Statistical significance was determined using the Mann-Whitney U-test.

In vitro angiogenesis

Sprouting and tube formation of EC was studied using cytodex-3 beads overgrown with EC in a 3-dimensional gel[17;18]. BCE were mixed with gelatin coated cytodex-3 microcarrier beads (Sigma, The Netherlands) at a concentration of 25 cells per bead and cultured for 72 hours in a tissue culture plate in RPMI-1640, supplemented with 20% HS, 2 mM L-glutamine, 50 ng/ml streptomycin, and 50 U/ml penicillin. The beads were spun down and re-suspended, in a concentration of 25 beads per 100 µl, in 8 volumes of vitrogen-100 (Collagen Corporation, Fermont, CA, USA), 1 volume 10× concentrated α-MEM (Life Technologies, Breda, The Netherlands), 1 volume 11.76 mg/ml sodium bicarbonate and 20 ng/ml bFGF. 100 µl of this mixture was suspended to each well of a 96 wells culture plate, after which gellation was allowed to take place at 37°C. After

gellation medium was applied on top of the gel containing 20 ng/ml bFGF, with or without BPI at concentrations as indicated and with or without 5 I.U./ml heparin. After 24 hours photographs were made. For quantification these images were analyzed using NIH image computer software. Statistical analysis was done using Mann-Whitney U test.

Chorioallantoic membrane (CAM) assay

Fertilized white leghorn chicken eggs were incubated for 3 days at 37°C and 60% humidity. On the third day a hatch was made in the egg shell. The eggs were further incubated at 37°C and 60% humidity until day 7. On this day a silicone ring was placed directly onto the CAM and was left to stabilize for 2 hours. Subsequently, the treatment of the CAMs started by daily addition of 65 µl of either vehicle (saline), 180 nM or 540 nM BPI dissolved in saline. On day 10 the CAMs were photographed. Quantification of vascularization was performed by enumeration of intersections with 3 concentric rings that were superimposed on the photographs.

Results

BPI inhibits endothelial cell growth

Potential angiogenic effects from BPI were first investigated *in vitro* using an EC proliferation assay with cultured human umbilical vein-derived endothelial cells (HUVEC). EC proliferation was measured by using a [^{3}H]-thymidine incorporation assay and by enumeration of living cells following three days of culture of HUVEC in the presence of various concentrations of BPI. Both bFGF-induced (20 ng/ml) (Figure 2.1) and spontaneous (not shown) EC proliferation were inhibited by BPI in a similar concentration dependent fashion. BPI inhibited 80% proliferation at 1.8 µM, whereas the half-maximal response was observed at about 250 nM (Figure 2.1A). To discriminate between cytostatic and cytotoxic effects, proliferation was also investigated by quantifying the number of living cells using the trypan blue dye-exclusion method. Similar results were found in both assays. In these experiments, bovine serum albumin (BSA) was used as a negative control and the angiogenesis inhibitor platelet factor 4 (PF4)[11] was used as a positive control. The dye-exclusion method indicated that BPI is cytotoxic for EC, whereas PF4 has been recognized as being cytostatic and not cytotoxic[19] (Figure 2.1B). Figure 2.1C demonstrates that HUVEC are at least an order of magnitude more sensitive to BPI than to PF4, in the [^{3}H]-thymidine incorporation assay.

In assays that determine the fraction of detached cells over time, a time dependent effect from BPI was observed. At a concentration of 1.8 µM, BPI induced a half maximal response within 24 hours and a maximal response after 48 hours (Figure 2.2). Neither PF4 nor BSA had any effect on EC attachment to the

matrix. In related experiments, BPI functioned similarly in cultures of human microvascular endothelium (MVEC) and of bovine microvascular EC (BCE).

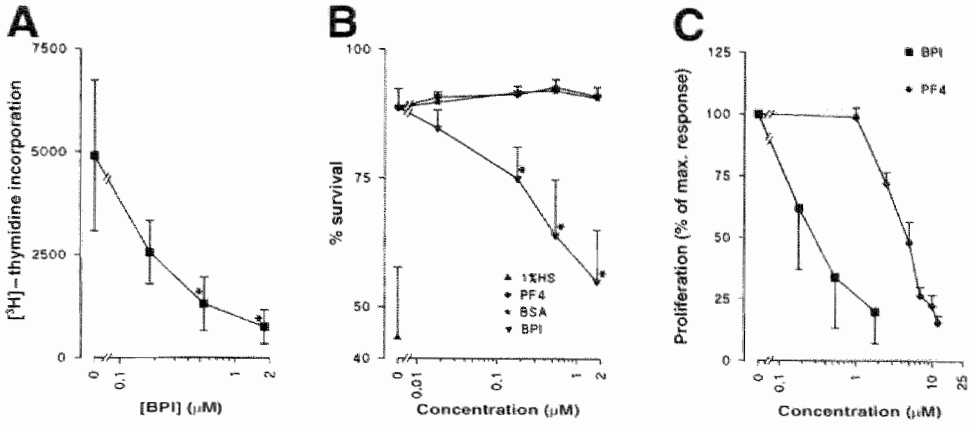

Figure 2.1 *BPI inhibits proliferation of HUVEC.*
A. Proliferation was measured after 72 hours of culture in the presence of 0.18, 0.54 or 1.8 μM BPI by analysis of [^{3}H]-thymidine incorporation. B. Cell death induced by BPI, PF4 or BSA, or after starvation by culture in the presence of low serum concentrations (1%), as determined by typan blue exclusion. C. Comparison of proliferation inhibition by BPI (n=4) and PF4 (n=9), plotted as percentage of thymidine incorporation under control conditions. In panel A and B the results of three independent experiments performed in triplicate are shown (*$p<0.05$).

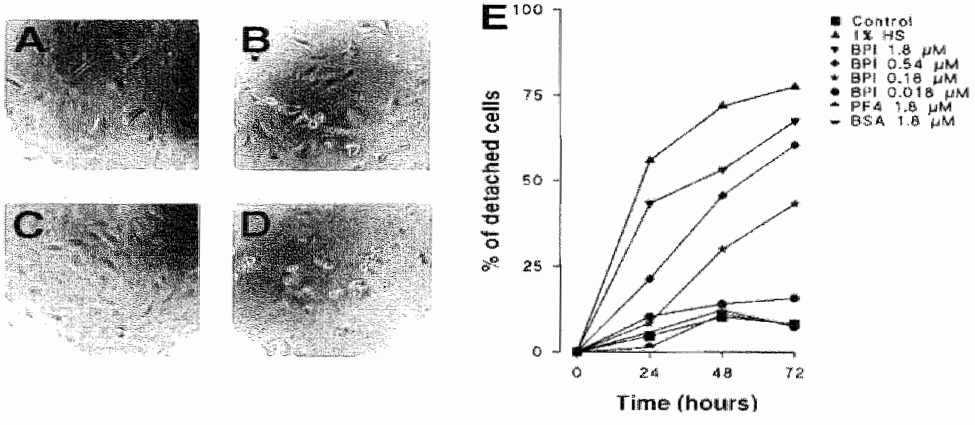

Figure 2.2 *BPI induces HUVEC to detach from the matrix.*
(A) control HUVEC after 24 hours, (B) HUVEC cultured with 1.8 μM BPI for 24 hours, (C) control HUVEC after 72 hours of culture, (D) HUVEC cultured with 1.8 μM BPI for 72 hours. After 24, 48 and 72 hours HUVEC were cultured under the indicated conditions and analysed under an inverted microscope for the ratio of detached cells. The mean results of three independent experiments are shown. For clarity, error bars have been left out, variation was less than 20% in all cases (E).

BPI induces apoptosis in endothelial cells

As shown in Figure 2.2, BPI induced the detachment and shrinkage of HUVEC. To evaluate whether BPI-induced cell death occurring in EC cultures involves induction of apoptosis, flow cytometric analysis was performed to assess the DNA profile. For three days, HUVEC were cultured in the presence of different concentrations of BPI, BSA and PF4, as well as under serum deprived conditions. Following cell harvesting, staining with propidium iodide and DNA extraction, FACS analysis was used to analyse the percentage of cells with a subdiploid DNA content. Having fragmented (subdiploid) DNA is indicative of apoptosis. For EC cultured in the presence of either BSA or PF4, the amount of cells in the subdiploid peak was comparable to that for HUVEC cultured under normal conditions. Serum deprivation basically starves cells and is known to trigger the apoptotic signal. In the presence of 1.8 μM BPI, a 10-fold to 13-fold elevation in apoptosis was observed as compared to that in control cultures. BPI concentrations of 180 nM, 540 nM and 1.8 μM induced apoptosis in 15%, 27% and 30% of the cells, respectively (Figure 2.3A). These levels are comparable to those observed in serum-starved cultures. BPI-induced apoptosis was confirmed on basis of cell morphology which was assessed in haematoxillin/eosin stained cytospin preparations (Table 2.1). A third line of evidence that confirmed induction of apoptosis by BPI is the use of ZVAD.fmk in cultures of HUVEC with BPI or serum deprivation. The addition of ZVAD.fmk at a concentration of 100 μM significantly inhibited the apoptosis induction by BPI with 71%±2%, whereas the cell detachment was not blocked by adding ZVAD.fmk. These results indicate the induction of apoptosis as a result of cell detachment and not vice versa. The effects of BPI are endothelial cell specific as indicated by the analysis of apoptosis induction by BPI in non-endothelial cells (the human adenocarcinoma cell-line Ls174T and freshly isolated human fibroblasts) as compared to HUVEC (Figure 2.3B).

Table 2.1 BPI induces nuclear fragmentation in HUVEC.

Condition	% of cells with fragmented nuclei	
	experiment 1	*experiment 2*
Control	5	3
BPI (1.8 μM)	79	69
PF4 (1.8 μM)	7	4
BSA (1.8 μM)	4	5
1% HS	99	71

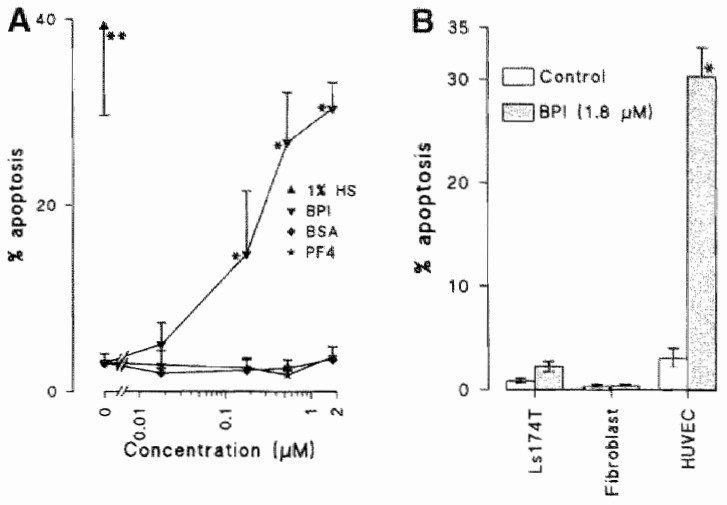

Figure 2.3 *BPI induces apoptosis in endothelial cells.*
(A) HUVEC were cultured for 3 days in normal culture medium, in medium with 1% human serum, or in medium containing BPI, BSA or PF4. After partial DNA extraction and propidium iodide staining, analysis of subdiploid EC under the different conditions was performed. (n=3, *p<0.025, **p<0.014). (B) Apoptosis induction by BPI is endothelial cell specific (n=3, *p<0.025). Percentage of subdiploid cells was also determined in Ls174T human colon adenocarcinoma (n=4) and in normal human foreskin derived fibroblasts (n=4).

Heparin blocks the apoptosis inducing activity of BPI

Many cytokines that are known to regulate angiogenesis, have the capacity to bind heparin. Since BPI is also known to bind heparin[8], the dependence of heparin on the anti-proliferative effect of BPI was investigated. Indeed, the addition of heparin to EC cultures inhibits the induction of apoptosis by BPI (Figure 2.4), suggesting that heparin does play a crucial role in the regulation of BPI-mediated inhibition of EC growth.

BPI inhibits in vitro angiogenesis

To further investigate the effects of BPI in subsequent steps of the angiogenic process, an *in vitro* angiogenesis assay was used in which gelatin-coated beads overgrown with EC were embedded in a semi-natural collagen matrix. Because EC of human macro- or microvessel origin cannot, or can only marginally, be induced to form tubes[20], bovine capillary EC were used in these studies. Under a stimulus of 20 ng/ml bFGF, EC were induced to sprout and to form tubes into the matrix. BPI efficiently inhibited BCE tube-formation in a concentration and time dependent fashion. At 1.8 μM, 81% inhibition was noted after only 24 hours. Half-maximal responses were observed at a concentration of 80 nM. Heparin at a concentration of 5 I.U./ml inhibits the BPI inhibition of *in vitro* angiogenesis by 56%±13%. PF4 also significantly inhibited angiogenesis by 77% at the same concentration (Figure 2.5).

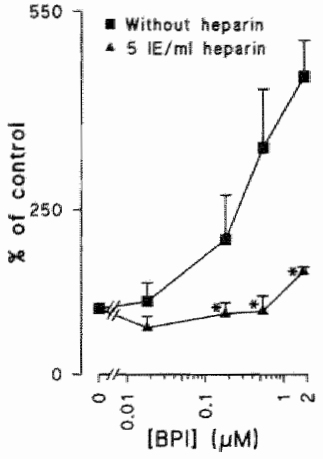

Figure 2.4 *Heparin prevents BPI induced apoptosis.*
HUVEC were cultured with or without heparin and different concentrations of BPI for 72 hours. After this period DNA fragmentation was analysed flow cytometrically (results shown are mean of three independent experiments ± SEM). Asterisks indicate significant difference ($p<0.05$) compared with cultures without heparin.

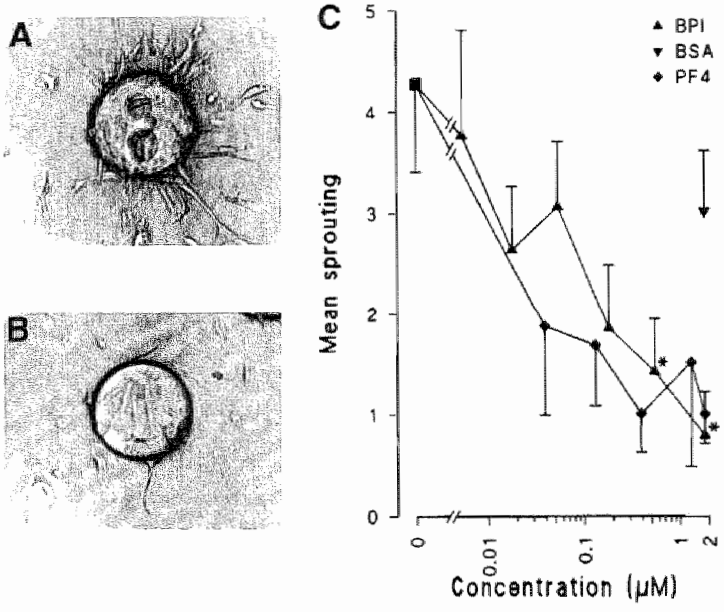

Figure 2.5 *BPI inhibits in vitro angiogenesis in a collagen matrix.*
BCE were cultured on gelatin coated Cytodex-3 beads in a collagen matrix. Sprouting was induced by addition of 20 ng/ml bFGF (panel A). Panel B shows sprouting of BCE in the presence of 20 ng/ml bFGF and 1.8 μM BPI. Quantification of results was performed by NIH-image software (n=3, *$p<0.013$).

BPI inhibits angiogenesis in vivo

To study inhibition of angiogenesis *in vivo*, the chick embryo chorioallantoic membrane (CAM)-assay was used. This assay measures developmental angiogenesis and is routinely used to obtain the first indication of angiogenic activity *in vivo*. CAMs were studied in intact eggs where windows of 1 x 1.5 cm were made at day 3 of development. Treatment was initiated at day 7. In CAMs treated daily with BPI between days 7 and 10 of development, a profound inhibition of microvessel formation was observed, whereas larger, preexisting vessels were apparently unaffected. Figure 2.6 shows the development of CAM vasculature at day 10 after fertilization and treatment with 180 nM (B), 540 nM (C) BPI or vehicle alone (A). Microvessel formation was inhibited by 42% at 180 nM and 48% at 540 nM. Treatment with vehicle alone resulted in the development of normal vasculature at day 10 similar to that observed in untreated CAMs.

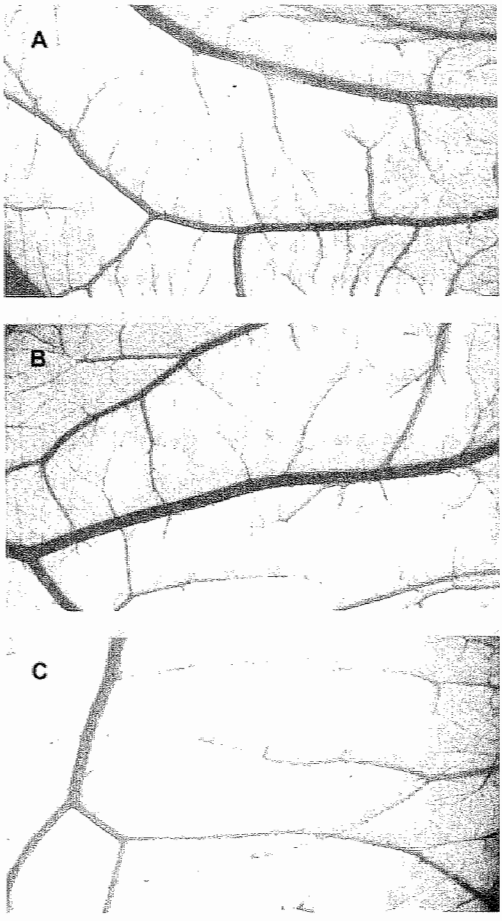

Figure 2.6 *BPI inhibits angiogenesis in the CAM assay.*
CAMs were treated for 4 days with vehicle alone (A), 180 nM BPI (B) or 540 nM BPI (C).

Discussion

A novel biological activity for BPI has been reported in this study. BPI has joined the list of anti-angiogenic agents which include PF4, angiostatin[21] and endostatin[22]. More and more angiogenesis inhibitors are continually being discovered and only a few will ever be used in therapeutic applications. BPI, aside from providing a potential treatment against sepsis, might also be used in the treatment of cancer and/or other agiogenesis-related pathological disorders. In its capacity as an anti-angiogenic, BPI works specifically on and is cytotoxic to EC, whereas both PF4 and endostatin, for example, are cytostatic, arresting EC in the cell cycle[11;19]. The concentration at which BPI is half-maximally effective at inhibiting *in vitro* angiogenesis, 80 nM, is considered to be quite low relative to that of many other inhibitors of angiogenesis[21;22] like PF4[11] that demonstrates a similar effectivity at a concentration about 10-fold higher in inhibiting proliferation, however the anti-angiogenic effects in the *in vitro* assay were quite similar[19].

These studies have been performed using native, full length BPI which has a molecular mass of 55 kD. The recombinant 23 kD N-terminal sequence from BPI has also been reported[8] as being functional in neutralizing the effects from bacterial endotoxin. It is unknown if it also demonstrates anti-angiogenic activity as does native BPI. A peptide derived from the β-sheet domain of that N-terminal sequence of BPI[23] also has the ability to neutralize LPS, albeit 50-fold less effective; yet demonstrates no effect on EC proliferation (data not presented). It may be that both ends of BPI are required for anti-angiogenic activity, although this remains to be tested.

EC cultured in the presence of native BPI detach from the matrix and undergo apoptosis. Induction of apoptosis can be a rapid event, for example, following signaling via death-domain containing receptors such as the Fas-Fas ligand interaction in leukocytes. Due to the fact that BPI-induced apoptosis in EC was demonstrated to occur as a rather delayed phenomenon, it is suggested that the effect from BPI occurs indirectly, perhaps via interaction(s) with some adhesion receptor(s) to inhibit attachment of EC to the extracellular matrix, thereby inducing apoptosis. The fact that the caspase inhibitor ZVAD.fmk does not prevent detachment of EC favors the view that the apoptosis is a result of detachment, which may explain the delayed appearance of apoptotic morphology. Induction of apoptosis has also been demonstrated with another anti-angiogenic agent, an anti-$\alpha_v\beta_3$-integrin antibody, which binds the EC surface $\alpha_v\beta_3$-integrin[24] to directly trigger apoptosis[25]. Future studies should elicit which cell surface molecule(s) functions as the receptor for BPI on the endothelium.

A specific question that arises from these data, is 'what, if any, is the relationship between the anti-bacterial and anti-angiogenic effects of BPI?' The former function involves interactions of BPI with the outer membrane of bacteria, whereas the latter function involves interactions presumably with cell surface

receptor(s) on EC. Part of the explanation may be that during bacterial infections and ongoing inflammatory responses, angiogenesis is heavily stimulated. Low levels of BPI have been found circulating in the blood of patients experiencing bacterially-induced or sterile inflammation[26]. Inflammatory cytokines such as TNF, interferon, TGF and interleukin-1 play an important role in this process. It is feasible that a feed-back mechanism for inhibition of angiogenesis after turning down the inflammation, may have evolved. These considerations are similar to those which have been discussed for PF4 in having a role in turning down angiogenesis after wound healing.

Comparing what is known about the molecular processes of BPI in these two biologically different functions may provide some clue to any possible relationship. Surprisingly, the LPS neutralizing effect from BPI occurs at a half-maximal concentration of about 20 nM[23], which is close to that reported here for its anti-angiogenic effect, 80 nM. BPI has a strong affinity for negatively charged lipids in membranes, and it therefore binds to the negatively charged LPS in the outer membrane of Gram negative bacteria. Through its network of positive charges, BPI is thought to bind sugar phosphate groups in LPS, thereby neutralizing this bacterial endotoxin[2;27]. This might also be a regulatory pathway of BPI, through its binding to negatively charged receptors in the EC membrane. In support of this idea, BPI is known to bind the polyanionic molecule heparin[8] via its cationic residues. BPI in fact contains three heparin binding sequences[8]. These amino acid sequences are similar in composition to many other angiogenic and angiostatic compounds[28].

Initially, chemical similarities and heparin binding abilities between BPI and vasoactive molecules like PF4, interleukin-8 (IL-8)[12;29] and other chemokines, urged us to investigate the effect of BPI on angiogenesis. Although these similarities with PF4 and IL-8 suggest shared mechanisms and/or receptors, the effect of BPI on EC is completely different. IL-8 acts as a angiogenic factor, and PF4, while also being angiostatic, does not induce apoptosis. These points suggest that BPI acts via a completely different mechanism and receptor(s). While the IL-8 receptors are known, a receptor for PF4 has also yet to be identified. Possibly the cell associated form of heparin, heparan sulfate and/or other interaction molecule containing some polyanionic carbohydrate moiety present in the extracellular matrix, could be the receptor and be bound by BPI. The possibility thus arises that BPI detaches endothelial cells by binding heparan sulfate on the extracellular matrix side of EC, thus preventing them from migrating through the extracellular matrix and leading to the induction of apoptosis. The binding of heparin by BPI can also have another role. Heparin is known to stabilize angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Heparin binding by BPI might compete for this stimulatory pathway. In addition, bFGF is normally stored in the extracellular matrix where it is bound to heparan sulfate, and can be released by heparin[30]. BPI, therefore, could block that process. This proposal is supported by the *in vitro* observation that BPI, in the presence of heparin, loses its anti-angiogenic activity.

In conclusion, BPI, produced in neutrophils, provides the human host with a natural defense against bacterial infection and also plays a role in the balance among angiogenic and angiostatic agents working together in the body. BPI, therefore, aside from providing a potential treatment against septic shock, might also be used in the treatment of cancer and/or other angiogenesis-related pathological disorders.

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Chapter 3

Anginex, a designed peptide that inhibits angiogenesis

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Abstract

Novel β-sheet-forming peptide 33mers, βpep peptides, have been designed by using a combination approach employing basic folding principles and incorporating short sequences from the β-sheet domains of anti-angiogenic proteins. One of these designed peptides (βpep-25) named anginex, is observed to be potently anti-angiogenic. Anginex specifically inhibits vascular endothelial cell proliferation and induces apoptosis in these cells as evidenced by flow cytometric detection of sub-diploid cells, TUNEL-analysis and cell morphology. Anginex also inhibits endothelial cell adhesion to and migration on different extracellular matrix components. Inhibition of angiogenesis *in vitro* is demonstrated in the sprout formation assay and *in vivo* in the chick embryo chorioallantoic membrane angiogenesis assay. Comparison of active and inactive βpep sequences allows structure-function relationships to be deduced. Five hydrophobic residues and two lysines appear to be crucial to activity. This is the first report of a designed peptide having a well-defined biological function as a novel cytokine, which may be an effective anti-angiogenic agent for therapeutic use against various pathological disorders such as neoplasia, rheumatoid arthritis, diabetic retinopathy and restenosis.

Introduction

Angiogenesis is crucial to numerous biological functions in the body, from normal processes like embryogenesis and wound healing to abnormal processes like tumor growth, arthritis, restenosis and diabetic retinopathy. The use of agents that can inhibit angiogenesis *in vitro* and *in vivo*, particularly in anti-tumor research[1,2], has indicated that anti-angiogenic therapy will be a promising therapeutic modality in the future. The search for angiogenic inhibitors has been focused on controlling two of the processes that promote angiogenesis: endothelial cell (EC) growth and adhesion[3,4] primarily because EC are more accessible than are other cells to pharmacologic agents delivered via the blood and EC are genetically stable and are not easily mutated into drug resistant variants. Most anti-angiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, which inhibit EC growth. This traditional approach has produced a number of anti-angiogenics, such as platelet factor 4 (PF4)[5], thrombospondin[6], tumor necrosis factor-α (TNFα, depending on its concentration)[7-9], interferon-γ inducible protein-10[10], angiostatin[11], endostatin and vasostatin[1,12] and bactericidal permeability-increasing (BPI) protein[13]. *In toto*, about forty anti-angiogenic agents, identified using various approaches, are currently known.

A survey of amino acid sequences from anti-angiogenic proteins reveals that they are compositionally similar, containing numerous hydrophobic and cationic residues. Comparison of the three-dimensional structures of several anti-angiogenic proteins, e.g. endostatin[14], PF4[15], tumor necrosis factor (TNF)[16] and BPI[17], provides a higher level of structural commonality in that they are comprised primarily of anti-parallel β-sheet structure. Another biological function common to at least two of these proteins, PF4[18] and BPI[19 and references therein], is their bactericidal activity. In fact, BPI was first identified and is better known for its ability to kill bacteria and to neutralize the bacterial endotoxin, lipopolysaccharide (LPS). These structural and compositional characteristics, therefore, appear to be functionally important to both anti-angiogenic and bactericidal activities which may suggest an evolutionary link. The selection of novel anti-angiogenic proteins or peptides may be guided by considering all these points: composition high in hydrophobic and cationic residues, anti-parallel β-sheet structure and bactericidal activity. In this regard, βpep peptides, which are designed amphipathic β-sheet-forming peptide 33mers[20,21] and are bactericidal[18], are obvious candidates.

βpep peptides were designed by using a combination approach employing basic folding principles and incorporating short sequences from the β-sheet domains of PF4, interleukin-8 (IL-8) and BPI[20]. Initially, the β-sheet domains from α-chemokines PF4 and IL-8 were used in part to create a presentation scaffold whose solubility in aqueous solution and structural stability were significantly enhanced using basic folding principles[20]. From the β-sheet domain of BPI (residues 82-108),

a few residues thought to be key to bactericidal activity[19] were designed into βpep peptides which then were shown to be, to varying degrees, bactericidal and capable of neutralizing LPS[18]. In BPI, this sequence of residues (82-108) is conformed in an amphipathic, anti-parallel β-sheet containing a large loop[17] and the solvent-exposed surface is cationic. NMR structural studies demonstrate that βpep peptides self-associate as dimers and tetramers whose monomer subunits are folded amphipathically in a three-stranded anti-parallel β-sheet motif [21]. Whereas the interior of the βpep β-sheet sandwich tetramer is composed primarily of aliphatic hydrophobic residues, the solvent-exposed surface displays a high net positive charge. The βpep peptide fold, although structurally similar, is different from those of the β-sheet domains of α-chemokines and BPI and is also similar to those folds found in a number of other β-sheet-structured cytokines[22]: interleukin-1 (IL-1)[23], tumor necrosis factor (TNF)[24], lymphotoxin (LT or TNF-β)[25], and transforming growth factor-β (TGF-β)[26].

With the hypothesis that βpep peptides might elicite angiostatic activity, the present study was aimed at assessing their ability to inhibit endothelial cell (EC) proliferation and angiogenesis. From a series of 30 βpep peptides, βpep-25 has been identified as a potent inhibitor of EC growth and angiogenesis. We call this peptide anginex. Anginex, which is more effective at inhibiting EC growth than PF4 and several other well-known angiogenesis inhibitors such as angiostatin, endostatin, AGM-1470 and thrombospondin-1, acts by specifically blocking adhesion and migration of angiogenically activated EC, leading to apoptosis and ultimately to inhibition of angiogenesis *in vitro* and *in vivo*. Sequence specificity is evidenced by results with a 91% homologous and 67% identical peptide, βpep-28, which displays no anti-angiogenic activity at the concentrations investigated. Anginex has the potential of becoming a potent therapeutic agent against various pathologic disorders like arthritis, restenosis, retinopathy and tumor growth.

Materials and methods

Peptide synthesis

Peptides were synthesized using a Milligen/Biosearch 9600 peptide solid-phase synthesizer using fluorenylmethoxycarbonyl chemistry. Lyophilized crude peptides were purified by preparative reversed-phase HPLC on a C18 column with an elution gradient of 0-60% acetonitrile with 0.1% trifluoroacetic acid in water. Pure peptides were subjected to gel filtration using a 1 x 9 cm Sephadex G25 column and pyrogen-free saline buffered with 0.008 M citrate phosphate buffer, pH 7.0, as elution buffer and used to confirm results obtained at every step of the study. The purity and composition of the peptides were verified by HPLC (Beckman Model 6300) analysis of amino acid composition of hydrolysates prepared by treating the peptides under argon in 6 N HCl for 24 hours at 110°C. The amino acid sequences of peptides were confirmed by N-terminal sequencing and mass spectrometry.

NMR

For NMR measurements, freeze-dried peptide was dissolved in H_2O/D_2O (90/10). Peptide concentration was 10 mg/ml. pH was adjusted by adding ml quantities of NaOD or DCl to the peptide sample. NMR spectra were acquired on a Varian UNITY Plus-800 NMR spectrometer. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans. Data were processed using VXNMR software directly on the spectrometer console or remotely on an SGI workstation.

2D-homonuclear magnetization transfer (HOHAHA) spectra, obtained by spin-locking with a MLEV-17 sequence[27] with a mixing time of 60 ms, were used to identify spin systems. NOESY experiments[28] were performed for conformational analysis. All 2D-NMR spectra were acquired in the TPPI[29] or States[30] phase sensitive mode. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments. 2D-NMR spectra were collected as 256 to 400 t1 experiments, each with 1k or 2k complex data points over a spectral width of 6 kHz in both dimensions with the carrier placed on the water resonance. For HOHAHA and NOESY spectra, normally 16 and 64 scans, respectively, were time averaged per t1 experiment. Data were processed directly on the spectrometer or offline using FELIX (Molecular Simulations, Inc.) on an SGI workstation. Data sets were multiplied in both dimensions by a 30-60 degree shifted sine-bell function and zero-filled to 1k in the t1 dimension prior to Fourier transformation.

Cells, cultures and reagents

Human umbilical vein derived EC (HUVEC) were harvested from normal human umbilical cords by perfusion with 0.125% trypsin/EDTA. Harvested HUVECs were cultured in gelatin coated tissue culture flasks and subcultured 1: 3 once a week in culture medium (RPMI-1640 with 20% human serum (HS), supplemented with 2 mM glutamine and 100 U/ml penicillin and 0,1 mg/ml streptomycin). Human microvascular EC (MVEC) were isolated from human foreskins and cultured in culture medium supplemented with endothelial cell growth supplement (ECGS, 15 µg/ml, Boehringer, Mannheim, Germany). Before use in *in vitro* assays MVEC were starved for 48 hours in culture medium without ECGS. Bovine capillary EC (BCE) were kindly provided by Dr. M. Furie, State University of New York, Stony Brook, USA. BCE were cultured in fibronectin coated tissue culture flasks in RPMI-1640 medium containing 10% FCS, glutamine and antibiotics. For isolation of recombinant PF4, the synthetic gene for human native PF4 was expressed as a non-fusion protein in *E. coli* (BL21) cells (Repligen Corp., Cambridge, MA). The protein was purified, cleaved and refolded essentially as previously described[32]. Purity was assessed by Coomassie staining of SDS-PAGE, analytical C4 reverse phase HPLC, and amino acid analysis. Typically,

several hundred milligrams of greater than 95% pure material was isolated from 100 g of starting material.

Proliferation measurement

EC proliferation was measured using a [^{3}H]-thymidine incorporation assay. EC were seeded at 5,000 cells/well in flatbottomed tissue culture plates and grown for 3 days, in the absence or presence of regulators, in culture medium. During the last 6 hours of the assay, the culture was pulsed with 0.5 μCi [methyl-^{3}H]thymidine/well. Results are expressed as the arithmetic mean counts per minute (cpm) of triplicate cultures.

Apoptosis measurement

HUVEC were cultured in fibronectin coated tissue culture flasks in culture medium[33]. Apoptosis was measured by determination of subdiploid cells after DNA extraction and subsequent staining with propidium iodide as described before[34]. In short, HUVEC were cultured for 3 days in the presence of 10 ng/ml bFGF and conditions as mentioned above. Serum deprivation of EC was used as a positive control for apoptosis. Cells were than harvested and subsequently fixed and permeabilized in 70% ethanol at -20°C. After at least 2 hours the cells were spun down and resuspended in DNA extraction buffer (45 mM Na_2HPO_4, 2.5 mM citric acid and 0.1% Triton X-100) for 20 minutes. Propidium iodide (PI) was added to a final concentration of 20 μg/ml and red log-scale fluorescence was analyzed on the FACS-calibur. Apoptosis was quantified as the percentage of cells with PI fluorescence below the fluorescence of cells in G_0/G_1 (diploid) phase of cell cycle. Cells with PI fluorescence less than 10% of cells in G_0/G_1 phase were regarded as cell fragments and were excluded for apoptosis measurements. TUNEL staining of cells was performed by immunocytochemistry on cytospin preparations of cultured cells, according to manufacturer's instructions (Mebstain Apoptosis kit, Immunotech, Marseille, France).

Cell adhesion and migration

HUVEC cultures were exposed to 100 μg/ml of βpep-25 (anginex) or culture medium (see above) alone. Following 24 hrs. in culture, cells were harvested by trypsinization and washed twice in culture medium. Cells were allowed to adher for time periods from 10 to 120 minutes in fresh 96-well culture plates coated with matrix components vitronectin, fibronectin, collagen or hyaluronic acid as described previously[33]. At these time points, non-adherent cells were washed away and adherent cells were enumerated under an inverted microscope. EC migration was measured in the wound assay. EC cultures were grown to confluence and wounds were made in the monolayer by removing cells with a sterile glass bar. Cultures were washed and the medium was replaced by fresh medium containing 10 ng/ml bFGF and incubated with or without inhibitors. Wound width was measured in triplicate cultures at four predefined locations at start and at 2, 4, 6, 8

and 24 hours after addition of the inhibitors. The student's t-test was used to determine statistical significance of observed differences.

FACS analysis of endothelial adhesion molecule expression

Immunofluorescence using indirect PE-conjugated reagents required three separate incubations. 1 x 10^5 HUVEC (cultured for 24 hours with 10 μg/ml βpep-25 or culture medium alone and harvested following incubation in 0.125% trypsin in PBS) were washed, fixed for 1 hour in 1% paraformaldehyde, resuspended in 20 μl appropriately diluted MAb and then incubated for 1 hour on ice. Subsequently, cells were washed twice in PBS/BSA (0.1%) and incubated for another 30 mins with biotinylated rabbit anti-mouse Ig (Dako, Glostrup, Denmark). After another two washings, cells were incubated with streptavidin-phycoerythrin conjugate (Dako). Stained cells were analyzed on a FACScalibur flowcytometer. For each sample, forward scatter (FSC), side scatter (SSC) and fluorescence signals from 5,000 cells were recorded. Data analysis was performed using CellQuest software (Becton Dickinson, Mountain View, CA).

In vitro angiogenesis assay

A semi-natural matrix of collagen type I was prepared by mixing 8 volumes vitrogen 100 (Collagen Corporation, Fermont, CA, USA) with 1 volume 10x concentrated MEM (Life Technologies) and 1 volume of sodium bicarbonate (11.76 mg/ml). The matrix was dispensed into culture plates and allowed to gel at 37°C. Confluent BCE were trypsinized and seeded on top of this collagen matrix. When cells were grown to a confluent monolayer, medium was changed by fresh medium, medium containing 25 ng/ml bFGF and the peptides or medium containing 25 ng/ml bFGF. Aside from experiments that added the angiogenesis inducer on top of the matrix, other experiments had breast cancer spheroids (Hs578T) embedded in the collagen matrix prior gelation in order to use a more realistic tumor-release model. Peptides were added to the culture simultaneously with the EC. In both experiments, after 48 hours of incubation the (sprouting) endothelial monolayers were photographed with a Zeiss inverted phase-contrast photo microscope. The amount of sprouting in each well (i.e. the total length of the sprouts) was quantified by the computer program NIH image[35]. The student's t-test was used to determine statistical significance of observed differences.

CAM-assay

Fertile Lohman selected white leghorn eggs were incubated for three days at 37°C and 55% relative humidity and rotated once every hour. At day 3, a rectangular window (1 x 2 cm) was made in the eggshell. The window was covered with tape to prevent dehydration. The window allowed undisturbed observation of the developing vasculature of the chorio-allantoic membrane (CAM). At day 7 a silastic ring (10 mm diameter) was placed on the CAM to allow local drug administration within the ring. Peptides βpep-25 (anginex) and βpep-28 dissolved in sterile saline (0.9% NaCl), were applied daily in aliquots of 65 μl from day 10 to

day 13. At day 14 the CAMs were photographed. Photographs were scanned and stereological principles were applied according to described methods. Three concentric rings were projected on the image. The number of intersections of rings and blood vessels was determined and used as a measure of vessel density. The student's t-test was used to determine statistical significance of observed differences.

Results

The amino acid sequences for βpep peptides are given in Figure 3.1 along with sequences from the β-sheet domains of IL-8, PF4 and BPI. These βpep-, IL-8, PF4- and BPI-peptides were tested for their ability to inhibit the proliferation of growth factor-induced (bFGF, 10 ng/ml) human umbilical vein endothelial cells (HUVECs) using the ^{3}H-thymidine incorporation assay. Whereas IL-8, PF4 and BPI peptides were inactive at the highest concentration tested (25 μM), a number of βpep peptides were inhibitory at both the 25 μM (100 μg/ml) and 2.5 μM (10 μg/ml) doses (Table 3.1). βpep-25 (anginex) was the most active, inhibiting about 50% EC growth at 2.5 μM. Similar results were found for VEGF-stimulated and spontaneous proliferation of EC. This being the case, the remainder of the paper is focused on the anti-angiogenic effects and mechanism of action of βpep-25.
Another βpep peptide, βpep-28, was chosen as a good negative control because it showed no EC growth inhibitory potency at 25 μM and was most homologous and identical to βpep-25. For assurance that βpep-25 and βpep-28 do indeed fold as β-sheets and to avoid the argument that indirect effects may account for activity differences, circular dichroism (CD) and ^{1}H NMR spectra were acquired with both peptides. βpep-28 shows well-defined, dispersed and downfield-shifted NH and αH resonances as expected for a β-sheet forming peptide (Figure 3.2) and as observed with other βpep peptides[18;20]. Since βpep peptides in general exist in solution in a monomer-dimer-tetramer equilibrium[20,36], pulsed field gradient (PFG) NMR self-diffusion measurements (not shown) were performed which indicate the presence of mostly tetramers and dimers for βpep-28 and βpep-25 at 2 mM peptide concentration. Even though CD data (not shown) demonstrate the presence of significant populations of β-sheet in both βpep-28 and βpep-25, aggregate exchange of βpep-25 monomers-dimers-tetramers falls within the intermediate exchange regime on the chemical shift time scale resulting in substantial resonance broadening (see Figure 3.2). This is particularly apparent for resonances arising from the less populated tetramer state. One very broad αH resonance envelope from the tetramer state, for example, is observed between 4.8 and 5.6 parts per million. Therefore, it is evident that βpep-25 forms weaker dimer-dimer subunit associations than does βpep-28. Structurally, NOESY data on βpep-28 (Figure 3.2, insert at upper right) show the presence of αH-αH NOEs indicative of the same anti-parallel β-sheet strand alignment as observed in other βpep peptides[21]. In fact, in all other βpep peptides which give similarly good NOESY spectra, this same overall β-sheet fold is conserved. Since βpep-25 is 91% sequentially homologous

and 67% sequentially identical to βpep-28, it is most probable that these two peptides also share the same overall β-sheet fold. Figure 3.2 (insert at upper left) gives the amino acid sequences for both βpep-25 and βpep-28, along with the β-sheet folding pattern for a monomer subunit within the tetramer state[21]. In the biological assays discussed below, the βpep peptide concentration is much lower than that used in these NMR structural studies. At these lower peptide concentrations, βpep tetramers dissociate into dimers and monomers[36]. Since CD spectra indicate that in both lower aggregate states some β-sheet structure is still present[36], this same folding pattern (Figure 3.2) may be conserved. However, this remains unknown.

BPI	A	N	I	K	I	S	G	K	W	K	A	Q	K	R	F	L	K	M	S	G	N	F	D	L	S	I	E	M	S	I	S	A	D
PF-4	R	H	I	T	S	L	E	V	I	K	A	G	P	H	S	P	T	A	Q	L	I	A	T	L	K	.	N	G	R	K	I	S	L	D
IL-8	K	F	I	K	E	L	R	V	I	E	S	G	P	H	S	A	N	T	E	I	I	V	K	L	S	.	.	D	G	R	E	L	S	L	D
βpep-1	K	F	I	V	T	L	R	V	I	K	A	G	P	H	S	P	T	A	Q	I	I	V	E	L	K	.	N	G	R	K	L	S	L	D
βpep-2	A	N	I	K	L	S	V	E	M	K	L	F	K	R	H	L	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-4	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-5	H	I	K	E	L	Q	V	K	W	K	A	Q	K	R	F	L	K	M	S	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-7	H	I	K	E	L	Q	V	K	M	K	A	Q	K	R	F	L	K	W	S	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-8	A	N	I	K	L	S	V	K	W	K	A	Q	K	R	F	L	K	M	S	I	N	V	D	L	S	.	D	G	R	E	L	S	L	D
βpep-11	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	N	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-14	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	L	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-15	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	L	K	L	N	.	D	G	R	E	L	S	L	D
βpep-16	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	A	K	L	N	.	D	G	R	E	L	S	L	D
βpep-17	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	L	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-18	S	I	Q	D	L	K	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-19	S	I	Q	K	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-20	S	I	Q	D	L	N	V	S	M	X	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-21	S	I	Q	D	L	N	V	S	L	K	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-22	S	I	Q	D	L	N	L	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-23	S	I	Q	D	L	K	V	S	L	N	L	F	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-24	S	I	Q	F	L	K	V	S	L	N	L	D	R	K	Q	A	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-25	A	N	I	K	L	S	V	Q	M	K	L	F	K	R	H	L	K	W	K	I	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-26	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	I	I	I	K	L	N	.	D	G	R	E	L	S	L	D
βpep-27	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	A	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-28	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	V	I	V	K	L	N	.	D	G	R	E	L	S	L	D
βpep-29	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	L	I	L	K	L	N	.	D	G	R	E	L	S	L	D
βpep-30	S	I	Q	D	L	N	V	S	M	K	L	F	R	K	Q	A	K	W	K	V	I	I	K	L	N	.	D	G	R	E	L	S	L	D

Figure 3.1 *Amino acid sequences are shown for βpep peptides, as well as those for the β-sheet domains from PF4, IL-8 and BPI.*
Sequences are aligned to the longer BPI peptide, and a gap has been left in the other sequences where one residue is absent relative to the BPI sequence.

Table 3.1 The library of βpep peptides tested for inhibition of EC proliferation.

βpep #	2.5 μM	25 μM
βpep-1	119[a] ± 15	79 ± 9
βpep-2	71 ± 17	8 ± 1
βpep-4	119 ± 4	100 ± 6
βpep-5	84 ± 9	29 ± 3
βpep-7	72 ± 8	13 ± 1
βpep-8	69 ± 4	6 ± 2
βpep-11	76 ± 10	39 ± 7
βpep-16	87 ± 3	103 ± 4
βpep-20	78 ± 11	34 ± 1
βpep-21	71 ± 6	22 ± 1
βpep-24	86 ± 9	26 ± 5
βpep-25	46 ± 2	12 ± 5
βpep-30	98 ± 9	76 ± 9

[a]HUVEC were cultured for 3 days with the indicated concentrations of βpep peptides. During the last 6 hours cultures were pulsed with 0.5 μCi/well of [^{3}H]-thymidine. Results (mean values of three independent experiments performed in triplicate) are percentages of counts per minute relative to control cultures without peptide.

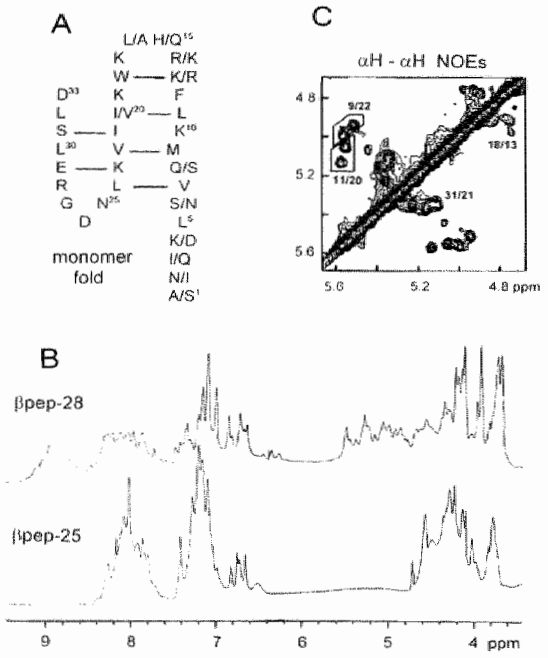

Figure 3.2 *The overall β-strand alignment in monomers is shown for βpep-25 and βpep-28 (upper left panel).* Vertical lines between strands indicate intra- and inter-monomer αH-αH alignments. The first of the two residues shown in the sequence as 'x/y' is for βpep-25 (x) and the second is for βpep-28 (y). 800 MHz ^{1}H NMR spectra are shown for βpep-25 (anginex) (bottom trace) and βpep-28 (middle trace) in H_2O/D_2O (90/10). The peptide concentration was 10 mg/ml in 20 mM potassium phosphate, pH 6.3, 40°C. Spectra were accumulated with 8 k data points over 6000 Hz sweep width and were processed with 2 Hz line broadening. Only the spectral region downfield from the water resonance is shown. In the NOESY spectrum at the upper right of the figure, several cross-peaks have been boxed-in and/or labeled to indicate intra- and inter- monomer αH-αH NOEs which have been used to indicate β-strand alignments. The boxes are labeled with two numbers separated by a slash to indicate which residues in the sequence are involved.

Growth-inhibitory effect from βpep-25 is specific for EC

To demonstrate that βpep-25 (anginex) specifically inhibits the growth of endothelial cells, Figure 3.3 gives a more complete βpep-25 dose response for growth-inhibitory potency against HUVECs (panel A), human microvascular EC (panel B), bovine capillary EC (panel C), normal human foreskin-derived fibroblasts (panel D) and breast cancer cell line HS578T (panel E).

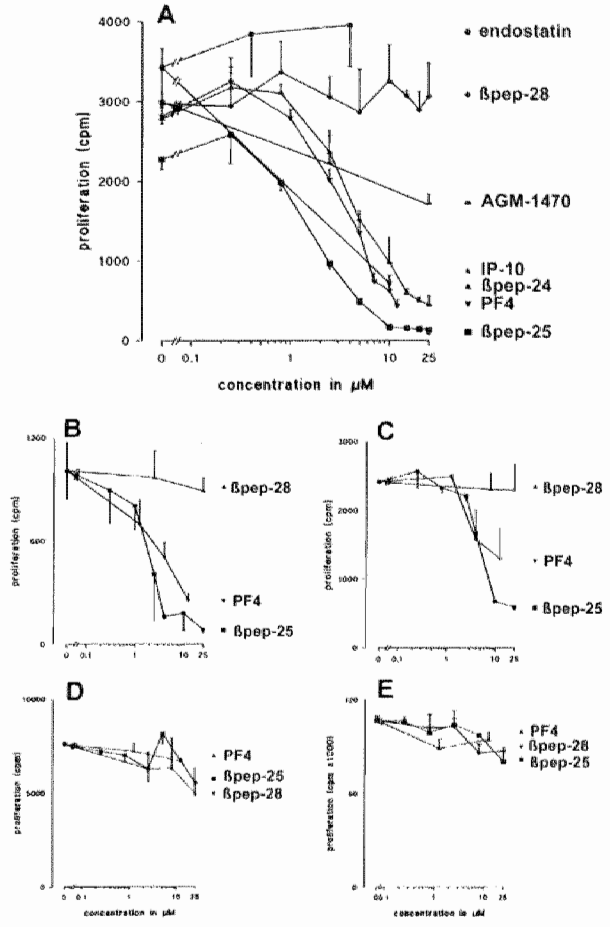

Figure 3.3 *βpep-25 specifically inhibits growth factor induced EC proliferation.*
Proliferation of bFGF-stimulated (10ng/ml) EC cultures is measured by quantification of ^{3}H-thymidine incorporation. Proliferation is expressed as mean counts per minute (cpm) of quadruplicate cultures in three independent experiments (±SEM). (A) Dose-response curves of βpep-25 and βpep-28 on bFGF-stimulated HUVEC proliferation. Dose-response curves of βpep-25 and βpep-28 on human microvascular EC (B), bovine capillary EC (C), normal human fibroblasts (D) and breast cancer cell line HS578T (E). Additional growth factors were not used to stimulate fibroblast and tumor cell cultures.

Human endometrium-derived fibroblasts, smooth muscle cells and PHA-stimulated peripheral blood leukocytes, as well as various other cancer cell lines were also tested (data not shown). βpep-25 was inhibitory only to the growth of EC. In this dose response curve, βpep-25 showed a half-maximal inhibitory effect at about 1.5 μM, close to the value of 2.5 μM reported above. In all cases, negative control peptide βpep-28 was ineffective as expected. In this same series of HUVEC proliferation experiments, various angiogenesis inhibitors demonstrated half-maximal inhibitory effects: endostatin (no effect detected), PF4 (4 μM), AGM-1470 (50 μM) and IFNγ-inducible protein-10 (3 μM). The absence of any inhibitory effect from endostatin was surprising. Addition of an equi-molar amount of low molecular weight heparin to the endostatin solution did cause a small inhibitory effect at 25 μM, but the effect was minimal at about 20% decrease in EC growth. In addition, the kinetics of βpep-25 inhibition of EC proliferation were the same as those for PF4 and these other angiogenic inhibitors, with half-maximal inhibition at 24-36 hours and maximal inhibition occurring after 2 to 3 days of culture (data not shown). Although results in Figure 3.3 were generated using bFGF-stimulated EC, essentially the same results were obtained using VEGF-stimulated and spontaneous proliferation.

βpep-25 mechanism of action

βpep-25 (anginex) is clearly inhibiting EC growth. This inhibition could occur via two main routes: 1) cytostatically, for example, by arresting EC in S-phase as observed for PF4[5], or 2) cytotoxically by directly or indirectly inducing EC death. Since detachment from the matrix, shrinkage and cell death was observed in EC proliferation assays (Figure 3.4), βpep-25 functions cytotoxically. The question then arose as to whether mortality was mediated by the induction of apoptosis. Apoptosis, most easily quantified by analysis of DNA fragmentation following DNA extraction and propidium iodide staining, was measured in HUVEC cultures with and without βpep-25 or βpep-28. Serum deprivation which is known to induce apoptosis in EC, was used as a positive control.Although already measurable after 24 hours, profound apoptosis- mediated DNA fragmentation by βpep-25 was observed after 48 hours of culture (Figure 3.4). Apoptosis reached maximal levels of approximately 80% to 90% of the cells after 3 days as indicated in the time-course insert given in the figure. The magnitude of the response was similar to the induction of apoptosis by starvation in medium containing low serum concentration. The negative control peptide, βpep-28, failed to induce apoptosis. As anticipated from earlier reports[5], native PF4 being cytostatic towards EC, also did not induce apoptosis. The apoptosis- inducing capacity of βpep-25 was confirmed by TUNEL analysis (Table 3.2) and by apoptotic morphology (not shown) on cytospin preparations of cultured cells. Although the number of apoptotic cells seems lower when assessed by the TUNEL assay, which is primarily due to the loss of cells in advanced stages of apoptosis in the cytospin procedure, these results confirm the induction of apoptosis by βpep-25.

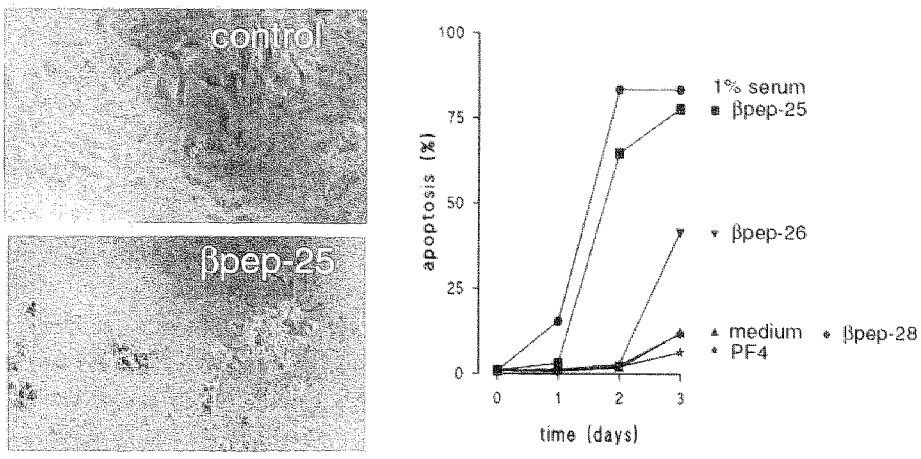

Figure 3.4 *βpep-25 induces apoptosis in cultured human endothelial cells.*
The left panels show HUVECs cultured for 2 days with or without 100 μg/ml βpep-25. Apoptosis induction was demonstrated by analysis of subdiploid cells after DNA extraction and staining with propidium iodide, and the panel at the right shows the time-response curve of HUVEC cultured for 3 days in the presence of either 1% serum (circles), βpep-25 (squares), βpep-28 (triangles), PF4 (asterisks) or medium alone (diamonds). Percentage of apoptosis is quantified as the percentage of sub-diploid cells as described in the Methods Section.

Apoptosis can be induced in a number of ways. For example, direct binding to the integrin receptor $\alpha_v\beta_3$, present on the cell surface of EC, can trigger the apoptotic cascade[37,38]. Alternatively, since EC must adhere to and migrate on components of the extracellular matrix during angiogenesis, prevention of EC attachment to matrix components (anoikis)[41,42] will induce apoptosis. These studies indicate that βpep-25 functions using the later pathway. Even though βpep-25 does not directly prevent attachment of HUVEC to matrix components, such as collagen, fibronectin, vitronectin or hyaluronan, which were immobilized on tissue culture plastic plates, exposure of HUVEC to 100 μg/ml βpep-25 for 24 hours, did lead to a significant suppression of adhesion to all matrix components tested. This is exemplified with vitronectin in Figure 3.5A. The negative control peptide βpep-28 had no effect. These results suggest that βpep-25 induces the down-regulation of adhesion receptors, rather than directly blocking interactions between matrix and EC adhesion molecules. This was confirmed by the observation that βpep-25 acts to down-regulate EC adhesion molecules $\alpha_2\beta_1$- , $\alpha_5\beta_1$- and $\alpha_v\beta_3$-integrins and CD44 (Figure 3.5C).

Table 3.2 TUNEL analysis of HUVECs.

	24 hours	3 days
medium	2.8[a]	1.0
25 μM PF4	3.6	1.5
25 μM βpep-28	1.9	0
25 μM βpep-25	4.9	37.4
starvation (1% serum)	26.2	NM

[a] percentage of TUNEL-positive HUVECs of a representative experiment out of three. NM, not measurable due to excessive progressed cell death.

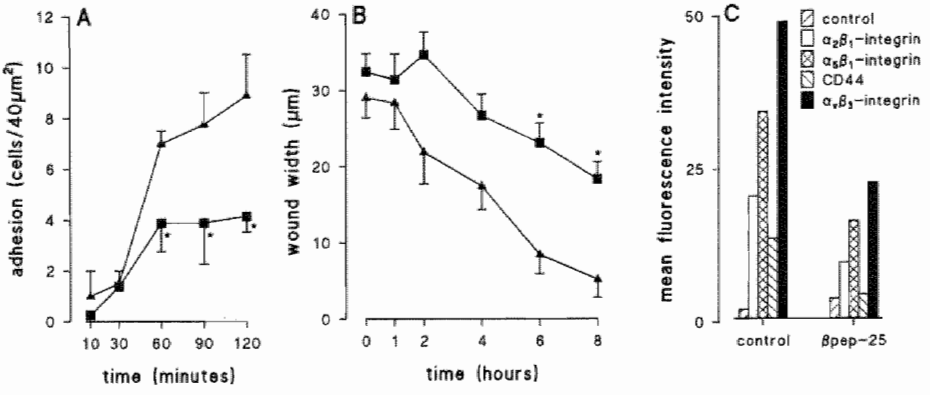

Figure 3.5 *βpep-25 inhibits EC adhesion and migration.*
A. HUVEC were cultured for 24 hours in the absence (triangles) or presence (squares) of 100 μg/ml βpep-25. Harvested cells were allowed to adhere to vitronectin coated tissue culture plastic. Non-adherent cells were washed away and percentages of adhered cells were determined by counting on an inverted microscope. **B.** Migration of EC was determined by wound assays. Confluent HUVEC cultures were wounded. Subsequently, wound width was measured at certain time-points. Migration of EC is inhibited by βpep-25 (triangles) as compared to untreated cells (squares). Mean results of three different experiments are shown (±SEM). Asterisks represent the statistical difference from control experiments performed in the absence of βpep-25 ($p < 0.01$). **C.** FACS analysis of endothelial adhesion molecules. HUVECs were exposed to 100 μg/ml βpep-25 or medium alone in control cultures for 24 hours. Data are from one representative experiment out of four and are expressed as mean fluorescence intensity from 5,000 cells.

To assess the effect of βpep-25 (anginex) on EC migration *in vitro*, the wound assay was used. On vitronectin-coated tissue culture plastic plates, EC migration was markedly inhibited, but not completely blocked, by the presence of βpep-25 (Figure 3.5B). Whereas in control wounds EC migrated to confluency within 8 hours, cultures treated with βpep-25 reached confluency only after at least 24 hours. Maximal inhibition of greater than 50% was observed after 2 hours. Again, βpep-28 demonstrated no effect on EC migration. All βpep peptides that inhibited

EC proliferation, had a proportional effect on EC migration, suggesting a direct correlation between these two processes (data not shown). EC migration on tissue culture plastic-coated plates with collagen, fibronectin, vitronectin or hyaluronan showed similar results (data not shown).

βpep-25 inhibits angiogenesis in vitro and in vivo

Since angiogenesis is a complex process which, aside from EC proliferation, depends on cell migration and differentiation, the effects of βpep peptides on angiogenesis were initially investigated in an *in vitro* collagen gel-based sprout formation assay. The same βpep peptides that inhibit EC proliferation also inhibit sprout formation. Again, of all βpep peptides, βpep-25 (anginex) demonstrated the greatest inhibition of angiogenesis. At 250 nM (1 μg/ml), βpep-25 inhibited greater than 40% of sprout formation, increasing to 80% at 25 μM (100 μg/ml, Figure 3.6). PF4 is also known to inhibit sprout formation[39]. The kinetics of inhibition for βpep-25 were the same as those for PF4. These results were obtained from experiments using bovine EC where sprout formation was induced with bFGF. Similar results were observed when human breast cancer spheroids were used in the gel as the angiogenic stimulus[40] to induce sprout formation in human foreskin-derived microvascular EC. In all assays where sprout formation was induced, βpep-25 was not cytotoxic for EC growing in monolayers, suggesting specificity for angiogenically-activated EC.

To study the inhibition of angiogenesis *in vivo*, the chick embryo chorio allantoic membrane (CAM)-assay was used. This assay which measures developmental angiogenesis, is routinely performed prior to use of an agent in *in vivo* tumor growth models. CAMs were studied in intact fertilized eggs where windows of 1 x 1.5 cm were made at day 3 of development. A flexible silicon ring into which compounds to be tested could be pipetted, was placed on the CAM at day 10, and treatment was initiated. In CAMs treated with βpep-25 (3 μg/ml) daily between days 10 and 13 of development, a profound inhibition of microvessel formation was observed, whereas larger, preexisting vessels were apparently unaffected. Figure 3.7 shows the development of CAM vasculature at day 14 after fertilization and treatment with βpep-25 or vehicle alone. Vessel counts (see Methods Section) after treatment with βpep-25 were 29.3 ± 3.5 versus 54.6 ± 4.1 for treatment with vehicle alone. Inhibition of vessel density was 46.3% (SD 5.9%; $n=5$; $p<0.002$). Treatment with βpep-28 or vehicle alone resulted in normal vascular development similar to that observed with untreated CAMs.

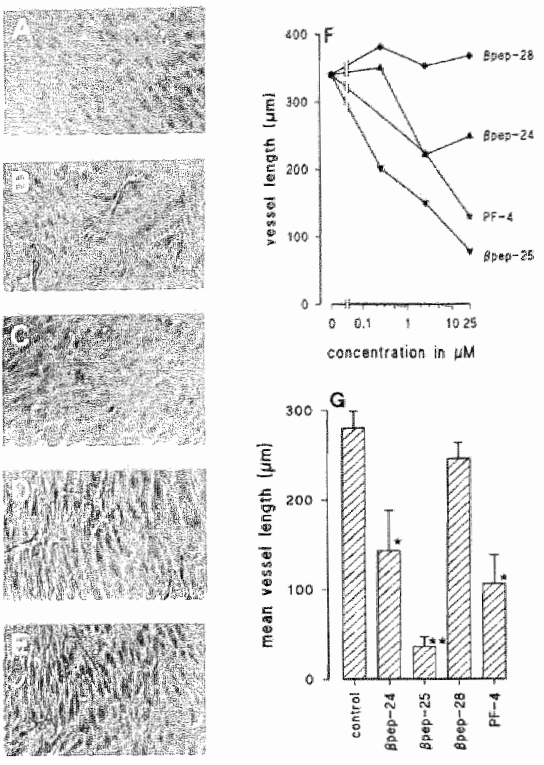

Figure 3.6 *In vitro angiogenesis is inhibited by βpep-25.*
Unstimulated BMEC (A) form tubes in a collagen gel upon stimulation with bFGF (B). This bFGF-induced stimulation is inhibited by βpep-25 (C), but not by βpep-28 (D). Moderate inhibition was found with βpep-24 (panel E). Panel F, showing the results of one representative experiment out of four, demonstrates that sprout formation is concentration dependent. Inhibition of sprouting at 25 µM (100 µg/ml), relative to PF4, is quantified in panel G. Asterisks represent statistical significance (mean sprouting ±SEM, n=6, *= $p<0.05$, **= $p<0.01$).

Discussion

The design of novel peptides is a powerful tool in the development of biologically active molecules. Anginex (βpep-25) is one such designer peptide with a clear biological function as a cytokine-like agent that specifically inhibits EC proliferation and angiogenesis. Because anginex induces apoptosis and causes cell death in EC, it is cytotoxic. Anginex is specific for angiogenically-activated EC. Resting EC as found in the confluent monolayer in the sprout formation assay, are apparently unaffected by the presence of anginex. This is important for any agent which may eventually be used therapeutically because it suggests that anginex should not act on quiescent EC in normal vasculature *in vivo*.

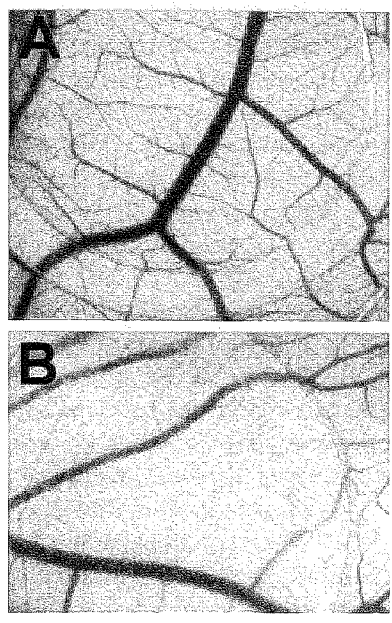

Figure 3.7 *Angiogenesis in the chick chorio-allantoic membrane is inhibited by βpep-25.* Development of vasculature in chick embryo chorio-allantoic membranes (CAMs) (vehicle alone in panel A) is inhibited by 3 μg/mL βpep-25 (panel B). CAMs treated with the control peptide βpep-28 developed similarly to untreated CAMs (vehicle alone). 65 μl quantities of 3 μg/ml solutions of peptide were added on days 10 through 13. Photographs represent CAM development on day 14. Scale: the silicon ring has a diameter of 10 mm.

Mechanistically, anginex inhibits EC adhesion to and migration on proteins components of the extracellular matrix, an event that subsequently induces apoptosis in the EC. Anginex, therefore, is cytotoxic towards EC. This apoptotic process is specific for angiogenically- activated EC and suggests a role for endothelial (cell surface) adhesion molecules (EAMs) that are upregulated during EC proliferation. Electron microscopic evidence demonstrates that the peptide is present on the matrix-binding ('abluminal') side of ECs after 24 hours of culture (data not shown). Integrins are one possibility for these EAMs. For example, $\alpha_v\beta_3$-integrin production is induced upon growth stimulation in EC and is therefore specifically expressed in vessels of angiogenically-stimulated tissues such as tumors[38]. Expression of CD44 and a number of other matrix binding integrins, mainly β_1-integrins, are also found to be elevated on angiogenically-activated EC[44;45]. However, since it was not possible to block the binding of anti-integrin ($\alpha_v\beta_3$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$) antibodies with anginex and *vice versa*, none of these integrins could be identified as the receptor for anginex. Binding to integrins often requires an RGD-motif on the peptide ligand. Anginex, however, does not contain the RGD tripeptide, but does contain the reverse tripeptide DGR. It is unknown whether this reverse sequence can promote RGD-type activity. Moreover, DGR is

also present in the negative control peptide βpep-28, supporting the conclusion that this sequence does not contribute to the angiostatic effect from anginex. RGD is not the only integrin binding sequence. For example, an endogenous fragment of MMP-2 called PEX which is also devoid of the RGD motif, was shown to bind to $\alpha_v\beta_3$-integrin[43]. Therefore, the absence of RGD does not preclude binding to integrins. In any event, since anginex affects adhesion to and migration on a number of extracellular matrix components, the peptide may in fact be interacting with more than one EAM.

The protein BPI functions similarly to anginex[13]. In itself, this initially may have been expected since the design of βpep peptides[20] was based in part on an amino acid sequence derived from β-sheet domain of BPI (residues 82-108). However, that same BPI sequence, made synthetically, is inactive as an antiangiogenic agent. Moreover, comparison of the anginex and BPI sequences (Figure 3.1) shows them to be homologous, yet quite different. In fact, anginex is sequentially closer to the β-sheet domain from PF4 also used in part in the design of βpep peptides. While the PF4 β-sheet domain sequence is also inactive, parent protein PF4 functions cytostatically, and not cytotoxically like anginex, by arresting EC in S-phase[5]. This, in turn, indicates that anginex and PF4 also function differently on the molecular level and emphasizes the idea that even though sequences are homologous, and even partly identical, bioactivities can be different. It may be that the activity of these peptides depends more on folded conformation than on sequence. For example, anginex forms β-sheet structure in aqueous solution, whereas the BPI peptide is unstructured random coil[18]. Quarternary structure is another factor which can modulate activity. Anginex, like various cytokines, e.g., α-chemokines and TNF, self-associates to form dimers and α-sandwich tetramers[21]. PF4 also forms dimers and tetramers in solution, yet PF4 is probably anti-angiogenically active as a monomer since at its normal concentration in the serum, biophysical studies indicate that PF4 would mostly be dissociated into monomers[50]. For IL-8, dimers which define the highest aggregation state, are considerably more stable than those for PF4; yet both monomers and dimers of IL-8 are biologically active[48,49]. Under physiologic conditions, anginex, like PF4, should exist mostly in the monomer state[36].

Identification of specific amino acid residues and their spatial relationships which are crucial to promoting anti-angiogenic effects at the level of molecular interactions, is one of the main goals among structural biologists and pharmaceutical chemists working to develop more effective anti-angiogenic agents. Nevertheless, in anti-angiogenic proteins such structure-activity relationships, i.e., specific residues and conformations which impart activity, are sorely needed, and even the analysis of high-resolution molecular structures of a number of anti-angiogenic proteins, e.g., endostatin[14], PF4[15] and BPI[17], have not provided this information. Working with a small peptide like anginex, however, allows more rapid assessment of some structure-activity relationships. By comparing these highly homologous βpep sequences (Figure 3.1) in terms of their relative ability to

inhibit EC proliferation, insight into which amino acid residues are required for activity, may be gleaned. First of all, since C-terminal residues 26 to 33 which were taken in large part from the β-sheet sequences of PF4 and IL-8, are identical in all βpep peptides, active and inactive, these residues are probably irrelevant to activity. Secondly, even though the N-terminal half of the most active βpep peptides is most like the β-sheet sequence from BPI, it can be made to be quite different with being detrimental to activity. For one of the most active peptides, βpep-8, residues identical to those in BPI are:
ANIK..S..KWKAQKRFLKMS..N..DLS... However, highly active βpep-2 and especially βpep-25, have much less sequence identity to BPI peptide 82-108, because the mid-portion of their sequences has been reversed relative to that found in βpep-8 and BPI: KWKAQKRFLKMS. Therefore, only the N-terminal stretch of residues ANIK..S... and the KWK tripeptide remains. The KWK tripeptide can also be excluded from imparting activity since it is present in most βpep peptides, and the ANIK..S sequence can be excluded because similarly active βpep-7, 11, 21 and 24 have completely different N-terminal sequences. Lastly, even though βpep-23 is sequentially most like inactive βpep-4, its anti-proliferative activity is quite good. In effect, therefore, nothing remains of the actual BPI β-sheet amino acid sequence (residues 82-108) used in part in the design of βpep peptides. Moreover, this same BPI sequence produced synthetically (Figure 3.1), is inactive at inhibiting EC proliferation, indicating that the BPI-derived sequence alone is not sufficient to promote this activity. Rather, it is the novel combination of amino acids within the βpep sequence that is paramount to activity. One additional reason for the presence of activity in these βpep peptides, is conformation. The BPI-derived sequence (residues 82-108) is completely random coil in solution, whereas all βpep peptides fold, to varying extents, as amphipathic β-sheets[18]. The novel βpep peptide design[20], therefore, has imposed certain sequential and conformational constraints in the context of a small peptide, which act to promote anti-proliferative and apoptotic activities against EC.

In the final analysis, residues in βpep-25 which emerge as being apparently crucial to anti-proliferative activity are L5, V7, K10, K17, I20, V22, L24. Assuming that the β-sheet fold of βpep-25 as shown in Figure 2 is the bioactive conformation, it is interesting to note that all five hydrophobic residues are proximal and are conformed on the hydrophobic face of the amphipathic β-sheet. The two lysines are positioned on the opposite amphiphilic face, lying diagonally from one another on strands 1 and 2. Although this does not prove that this is the bioactive conformation or that these amino acid residues are the most important, it is suggestive. As structure-function relationships in other anti-angiogenic proteins and peptides become known, it will be interesting to see if this is a common feature among anti-angiogenic proteins.

Conclusions

A number of βpep peptides can specifically inhibit proliferation in EC. Anginex (βpep-25) is the most potent of these, and functions by inhibiting EC adhesion and migration thereby inducing apoptosis. This cytotoxic, specific for angiogenically-activated EC, forms the basis for the angiostatic activity of the peptide. Comparison between active and inactive βpep peptide sequences has suggested that several hydrophobic and two lysine residues may be crucial in promoting these activites. This novel peptide anginex, therefore, makes for a potentially good anti-angiogenic therapeutic agent which may be capable of controlling various pathological disorders like tumor growth, rheumatoid arthritis, restenosis, and diabetic retinopathy.

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Chapter 4

Anginex inhibits angiogenesis and tumor growth in mice

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Abstract

The recently described angiogenesis inhibitor anginex (βpep-25) has previously been shown to inhibit proliferation and induce apoptosis in angiogenically activated human endothelial cells (ECs). In order to investigate the potential of anginex to be used for cancer treatment, the efficacy was studied in *in vivo* mouse models. Anginex was tested for anti-tumor effects in the B16F10 melanoma model in C57BL/6 mice and showed dose dependent inhibition of tumor growth by 58% at 0.2 mg/day when continuously administered using osmotic minipumps. The anti-tumor effects were induced by inhibition of angiogenesis as measured by vascular density and magnetic resonance imaging (MRI) analysis. *In vivo* anti-angiogenic activity of anginex was also demonstrated in the chick embryo chorioallatoid membrane assay and the matrigel plug assay in mice. The mechanism of action was studied using the mouse EC lines SVEC4-10 and TME and freshly isolated mouse EC. Anginex inhibited proliferation by 74% in TME and by 50% in SVEC4-10 and induced apoptosis in these cell lines as measured by subdiploid peak analysis. When SVEC4-10 or TME were cultured for 3 days in the presence of 75 μM anginex, 8% and 12% of the cells, respectively, revealed an apoptotic subdiploid DNA profile. By using FACS analysis of DNA fragmentation in CD31 positive cells, apoptosis induction by anginex was also demonstrated in freshly isolated and activated EC derived from mouse lung and heart tissue. To study regulation of migratory activity of EC, the wound assay was used. Significant inhibition of migration could be observed 4 hours after wounding the monolayer. At 75 μM, anginex potently inhibited up to 90% of migration of both SVEC4-10 and TME cells when measured after 24 hours. The latter may suggest that specific cell adhesion processes may be regulated by anginex. The current study revealed promising results for the development of anginex for use in patients with cancer.

Introduction

Angiogenesis is crucial to numerous biological bodily functions from normal processes like embryogenesis and wound healing to abnormal processes like tumor growth, arthritis, restenosis and diabetic retinopathy[1;2]. The search for angiogenesis inhibitors, which could be therapeutically useful, has been mainly concentrated on controlling two of the processes promoting angiogenesis: endothelial cell (EC) growth and cell adhesion[3;4]. Targeting drugs to ECs is considered a potential anti-cancer strategy primarily because ECs are more accessible than are other cells to pharmacologic agents delivered via the blood and because ECs are genetically stable and thus are not easily mutated into drug resistant variants. Most anti-angiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, which inhibit EC growth. This traditional approach has produced a number of anti-angiogenic agents, such as platelet factor 4 (PF4)[5], thrombospondin-1[6], interferon-γ inducible protein-10[7], angiostatin[8], endostatin[9], vasostatin[10] and bactericidal/ permeability increasing (BPI) protein[11]. The use of anti-angiogenic agents in *in vitro* and *in vivo* studies, particularly those applied to anti-tumor research[12], has strongly suggested that anti-angiogenic therapy will be a promising therapeutic modality in the future.

In search of novel angiostatic molecules, we have designed a β-sheet forming peptide, anginex (βpep-25), which is a potent inhibitor of angiogenesis that functions by blocking adhesion and migration of activated EC[13-15]. The aim of the present study was to assess efficacy of anginex at (i) inhibition of angiogenesis in mice and (ii) inhibition of tumor growth in mice. To test this, the peptide was tested in a syngenic mouse tumor model of B16F10 melanoma in C57BL/6 mice. Mechanistic studies were performed in *in vitro* models using mouse EC cell lines and freshly isolated microvascular ECs.

Materials and methods

Cell cultures and isolation procedures

Murine EC cell lines SVEC4-10 and TME were purchased from ATCC. Both cell lines were cultured in DMEM (life technologies, Breda, the Netherelands) supplemented with 10% FCS (BioWhittaker, Verviers, Belgium), 2 mM HEPES (Serva, Heidelberg, Germany), 50 IU/ml penicillin (ICN, Aurora, Ohio) and 50 IU/ml streptomycin (Seva, Heideberg, Germany), in gelatin (Merch, Darmstadt, Germany) coated tissue culture flasks (Costar, Corning, NY). The cell lines were subcultured 1:8. B16F10 melanoma cells were cultured in MEM with Hank's salts, 10% FCS, antibiotics, MEM-vitamins, glutamine, non essential amino acids, sodium pyruvate and $NaHCO_3$.

For some experiments mouse ECs were isolated from heart and lung tissues of C57BL/6 mice. The organs were taken out, minced and subsequently incubated in collagenase (Life technologies, Breda, the Netherlands) and dispase (Life technologies, Breda, the Netherlands) (both 1 mg/ml) for 1 hour at 37°C. After 30 minutes DNAse (Sigma Aldrich, Steinheim, Germany) was added to the mixture. After this incubation time the tissue was sieved and centrifuged at 1500 rpm for 10 minutes. The pellet was resuspended in culture medium (DMEM, 20% FCS, 2 mM L-glutamine, 2 mM sodiumpyruvaat, 20 mM HEPES, 1% non essential amino acids, antibiotics, 1 IU/100 ml heparin, 100 µg/ml ECGS). The cells were seeded in 25 cm^2 culture flasks coated with fibronectin. After 1 hour of adherence the non-adhered cells were decanted and the adherent cells were then cultured for three days in culture medium or medium supplemented with 10 ng/ml bFGF (Sanvertech, Heerhugowaard, the Netherlands) with or without 25 µM anginex. For FACS analysis ECs were identified by indirect staining with phycoerythrin labelled rat anti-mouse CD31 (Pharmingen, BD, Woerden, the Netherlands).

Apoptosis measurement

Cells were cultured for 3 days on a fibronectin coat in the presence or absence of 25 µM anginex. After this period the cells were harvested with trypsin (0.125%) (DIFCO, Detroit, Michigan) and fixed for two hours in 70% ethanol at -20°C. The cells were subsequently centrifuged at 1500 rpm for 5 minutes and resuspended in DNA extraction buffer (89 volumes 0.05 M $Na_2HPO_4.2H_2O$, 10 volumes 0.025 M citric acid, 1 volume 100% triton-X100, pH 7.4) and incubated for 20' at 37°C. After the incubation propidium iodide (PI) (Brunschwig Chemie, Amsterdam, the Netherlands) was added to a final concentration of 20 µg/ml and the DNA profile was directly analysed with the FACScalibur.

For assessment of apoptosis in freshly isolated ECs, CD31 staining was performed before the incubation in PI.

Proliferation measurement

Cells were seeded at 3,000 cells per well in a 96-well culture plate and allowed to adhere for 2 hours to a fibronectin (1mg/ml, 2 hours at RT) coat. After adherence the medium was replaced by medium containing a concentration range anginex and after 3 days the culture was pulsed for 6 hours with 0.3 µCi [methyl-^{3}H]-thymidine (Amersham Life Science, 's Hertogenbosch, the Netherlands)/well. Activity was measured using liquid scintillation. Measurements were done in triplicate.

Migration measurement

SVEC4-10 and TME were cultured on a 1 mg/ml fibronectin coat in a 24-well tissue culture plate. Cells were grown for 3 days until confluent. When confluent, a wound was made in the well using a blunt glass pipette. The medium was replaced with medium containing 0, 0.75, 2.5, 7.5, 25 or 75 µM anginex and at 0, 2, 4, 6, 8 and 24 hours the wound width was measured.

Chorioallantoic membrane assay

Fertilized eggs of Lohman-selected White Leghorns were incubated for 3 days at 37°C, 55% relative air humidity, while being rotated every hour. At day 3 a rectangular window (1 × 1.5 cm) was made in the shell. Two ml of albumin were withdrawn through the blunt end of the egg. The window was covered with scotch tape to prevent dehydration and the eggs were further incubated without rotation until day 10. At day 10 a silicone ring was placed on the chorioallantoic membrane (CAM) and inside this ring daily treatments were applied (65 µl of different concentrations of anginex or control peptide βpep-28). At day 14 photographs of the area at 6 times magnification were taken using a Wild M8 stereomicroscope equipped with a Nikon F301 camera. The vascular density index was analyzed by projection of 4 concentric rings on the image of the CAM and enumeration of intersections with blood vessels.

Matrigel plug assay

C57BL/6 mice were injected subcutaneously on the flanks with 500 µl matrigel (BD Pharmingen, Heidelberg, Germany) with 50 ng/ml bFGF with or without 2 mg/ml anginex. The matrigel plugs were taken out after 10 days and frozen. Sections were made and stained with rat anti-mouse CD31 antibody and peroxidase labeled goat anti-rat Ig polyclonal antibody.

Tumor model

The animal experiments were approved by an ethical review committee. C57BL/6 inbred mice were 6 weeks at the start of the experiment. At day 0, $2x10^5$ B16F10 cells (provided by Dr J. Fidler, Texas[16]) were inoculated subcutaneously on the right flank. On the left flank an Alzet osmotic minipump (Durect, Cupertino, CA) was placed at the same day. The Alzet pumps administered 0, 0.05, 0.2, or 0.4 mg/day of anginex, or 0.4 mg/day βpep-28 or BSA, dissolved in 0.9% NaCl solution (saline). TNP-470/AGM-1470 (kindly provided by Takeda Chemical Industries, Osaka, Japan), which was used as a positive control angiostatic compound, was administered by i.p. injections of 60 mg/kg every 2 days [17], as of day 9 after inoculation of the cells. Between day 6 and 9 the tumors became visible in all mice and tumors were measured daily. Tumor volumes were calculated as follows: $width^2 \times length \times 0.52$. Since we observed in this model that the first mice died of the disease shortly after day 16, the experiments were terminated on day 16. Statistical differences in tumor growth curves were analyzed using the two way ANOVA test.

On day 16, one hour before being sacrificed, 5 control mice (3 BSA and 2 saline treated) and 3 anginex treated mice were selected for magnetic resonance imaging (MRI). Mice with approximately equal tumor volumes were anesthesized (Ketamine 100 mg/kg and Xylazine 10 mg/kg, subcutaneous) and MRI measurements were done with a 5 cm diameter high-resolution surface coil at 1.5 Tesla (ACS-NT, Philips Medical Systems, Best, The Netherlands). The MRI

protocol comprised a T_2-weighted 2D-TSE sequence (TR/TE 3427/150 ms, TF 25) and T_1-weighted 3D-FFE (TR/TE 50/7 ms, flip 35°, voxel 0.5x0.5x2 mm^3) pre- and post contrast enhancement (Gd-DTPA 0.015 mmol/kg; Magnevist, Schering, Berlin, Germany). After the measurements the mice were sacrificed and the tumors were frozen in liquid nitrogen for histological analysis. Vessels were stained with rat anti-mouse CD31 and peroxidase labeled goat anti-rat Ig. The microvessel density (MVD) was evaluated as described previously[18]. In short, two independent observers assessed MVD by counting the total amount of blood vessels in three high-power fields (100x) randomly selected within a section. MVD is expressed as mean number of vessels per mm^2.

Results

Anginex inhibits proliferation of and induces apoptosis in mouse ECs

Proliferation of ECs is key in the process of angiogenesis. The influence of anginex on proliferation of mouse EC was analyzed using the EC lines SVEC4-10 and TME (Figure 4.1A). After three days of incubation with 75 μM anginex the proliferation was inhibited by 50% and 74%, respectively. To investigate whether growth inhibition of mouse EC was associated with the induction of apoptosis in these cells, flow cytometric analysis of DNA fragmentation was performed after culturing in the presence of anginex (Figure 4.1B). Apoptosis induction by anginex in both TME and SVEC4-10 was observed to be concentration dependent with maximal apoptosis induction in both cell lines being found to occur at 75 μM.

When primary cultures of mouse ECs isolated from lungs and heart of mice and cultured for 3 days on a fibronectin coat with or without bFGF and in the presence or absence of 25 μM anginex, similar results were observed. Heart EC cultured under normal conditions showed a cobble stone-like morphology, with detachment and apoptotic morphology being observed after exposure to anginex. Quantification of anginex-induced apoptosis in these EC is illustrated in Figure 4.1C.

Anginex inhibits the migration of mouse ECs

Aside from EC growth, migration also plays a role in the process of angiogenesis. To explore the effect of anginex on EC migration, the wound assay was used. Addition of anginex to wounded confluent monolayers of SVEC4-10 and TME, caused migration of these cells to be inhibited in a dose dependent manner. Figures 4.2A-D show TME cultures of control (A and C) and anginex-treated (B and D) cells, 8 hours (A and B) and 24 hours (C and D) after wounding. At a concentration of 75 μM anginex, 91% and 88% inhibition was observed 24 hours after wounding the monolayer of TME cells (Figure 2E) and SVEC4-10 (Figure 4.2F) cells, respectively.

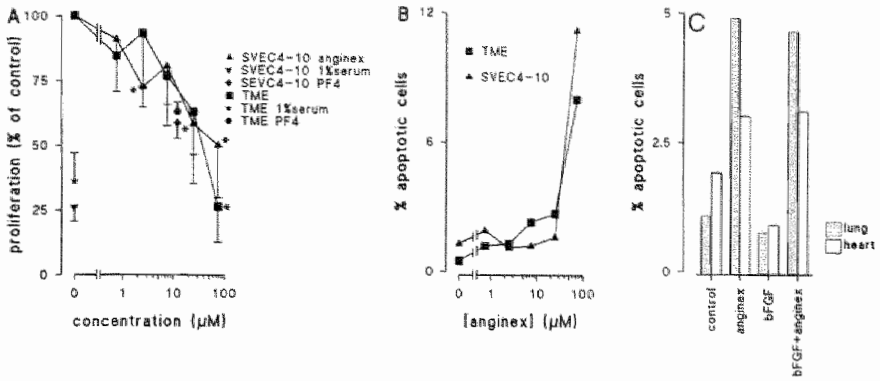

Figure 4.1 *Anginex inhibits proliferation and induces apoptosis in mouse EC.*
SVEC4-10 and TME were cultured for 3 days in the presence of a concentration range of anginex. Proliferation was measured by [^{3}H]-thymidine incorporation. Panel A shows the mean data of four independent experiments (± SE, * $p<0.05$). Panel B shows anginex induced apoptosis as measured by assessment of subdiploid cells after DNA staining with propidium iodide. Results of one representative experiment out of four are shown. C Anginex induces apoptosis in EC isolated from mouse tissues. Single cell suspensions were made from heart and lungs of C57BL/6 mice and cultured for 3 days on fibronectin coated tissue culture plates in the absence or presence of 10 ng/ml bFGF and with or without anginex. Subdiploid PI staining cells after DNA extraction was determined in the CD31 expressing subset of cells using flow cytometry.

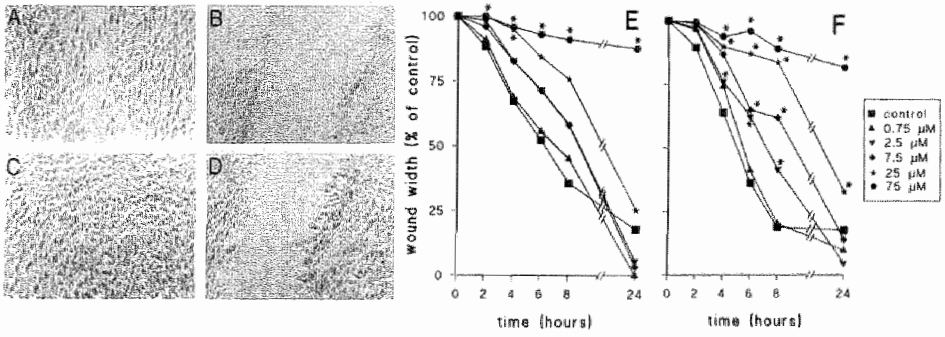

Figure 4.2 *Anginex inhibits migration of SVEC4-10 and TME.*
SVEC4-10 and TME were grown until confluence was reached, after which the monolayer was wounded and medium was replaced by medium with 10 ng/ml bFGF or medium supplemented with anginex in different concentrations. Panel A shows the control culture of TME at 8 hours and C at 24 hours. Panel B shows the same culture with 75 μM anginex after 8 hours and D after 24 hours. Panel E shows the quantification of anginex induced inhibition of migration of TME. Panel F shows the migration inhibition in SVEC4-10. Results are presented as mean values of five different experiments. SE values are left out for sake of clarity and were typically smaller than 10%. *$p<0.05$.

Anginex inhibits angiogenesis in in vivo models

In order to assay whether angiogenesis *in vivo* is modulated by anginex, two models were used: the chorioallantoic membrane (CAM) assay in the chick embryo and the matrigel plug assay in nude mice. In the CAM-assay, which is a model usually used to determine developmental angiogenesis, anginex inhibited angiogenesis in a concentration dependent way (Figure 4.3A-E). The maximal response of 50% angiogenesis inhibition was reached at 50 μM anginex. Responses, however, were already visible at 0.075 μM concentrations, whereas treatment with the negative control peptide βpep-28, which only differs in a few amino acids from anginex, did not show any activity (Figure 4.3F).

In the matrigel plug assay, the presence of anginex markedly inhibited bFGF-induced angiogenesis. This was demonstrated by CD31 staining of microvessels in the matrigel plug (Figure 4.4).

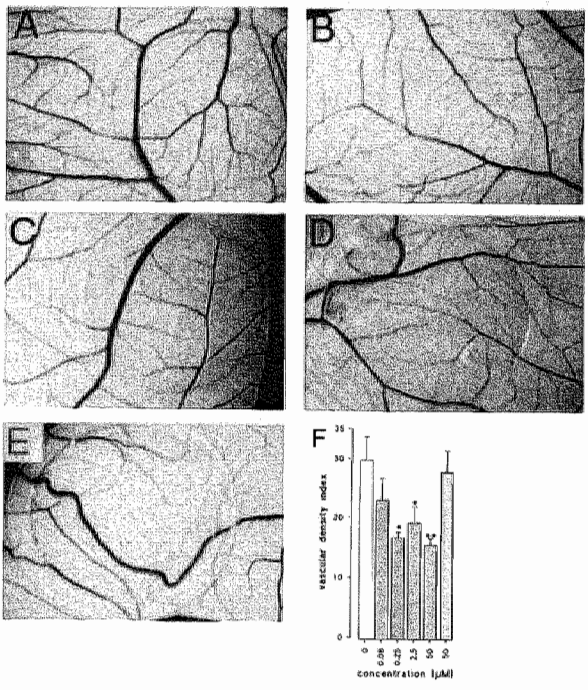

Figure 4.3 *Anginex inhibits in vivo angiogenesis in the CAM in a concentration dependent manner.*
The chorioallantoic membrane was treated with saline (A), 0.075 (B), 0.25 (C), 2.5 (D) or 50 μM anginex (E). The vascular density index of the CAMs was quantified (see M&M section) by projection of 4 concentric rings over the image and counting the number of intersections with vessels. Panel F shows the quantification of vessel density of CAMs of 5 saline control embryos (white), 6 CAMs treated with 0.08 μM, 3 CAMs treated with 0.25 μM, 5 CAMs treated with 2.5 μM and 6 CAMs treated with 50 μM anginex (dashed bars) and 5 CAMs treated with 50 μM βpep-28 (mean number intersecting vessels/10 mm, ±SE, * $p<0.01$, ** $p<0.0005$).

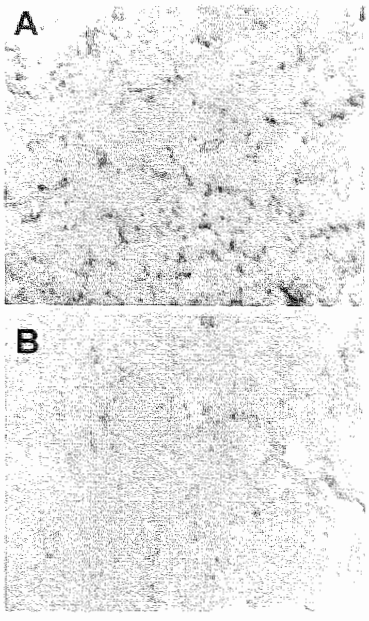

Figure 4.4 *Anginex inhibits bFGF induced angiogenesis in a subcutaneous matrigel plug in C57BL/6 mice.*
bFGF at 50 ng/ml matrigel induces angiogenesis in mice (A). When 2 mg/ml anginex is added to the matrigel plug angiogenesis is inhibited (B).

Anginex inhibits tumor growth and angiogenesis in a melanoma mouse model

Since it became evident that mouse EC in culture were sensitive to anginex treatment, the anti-tumor effect of anginex was examined in the mouse melanoma model B16F10 in C57BL/6 mice. Treatment of mice using various doses of anginex was initiated at the time of inoculation with tumors cells, and a profound dose-dependent inhibition of tumor growth by anginex was observed. At a dose of 0.2 mg/day, administered by continuous s.c. infusion using osmotic minipumps, tumor growth was significantly inhibited by 58% as compared to that in control mice treated with saline alone. While treatment of mice with 0.4 mg/day did not lead to a significant increase in efficacy (63% inhibition), a lower dose of 0.05 mg/day inhibited tumor growth by only 36% (Figure 4.5). TNP-470, used in a previously optimized treatment regimen[17], was used in these experiments as positive control (approx. 60% inhibition) to demonstrate that angiogenesis inhibition can inhibit tumor growth in this model. Anginex treatment showed no toxicity in these animals as assessed by macroscopic and behavioral (grooming) determinants and body weight, as well as by histological evaluation of the organs and measurement of hematocrit (not shown).

To determine whether the tumor inhibitory effect by anginex was the result of angiogenesis inhibition, tumors were immunohistochemically screened for microvessel density using anti-CD31 antibody. Anginex treatment resulted in a

lower vessel density in the tumor of approximately 50%, relative to control tumors (Figure 4.6).

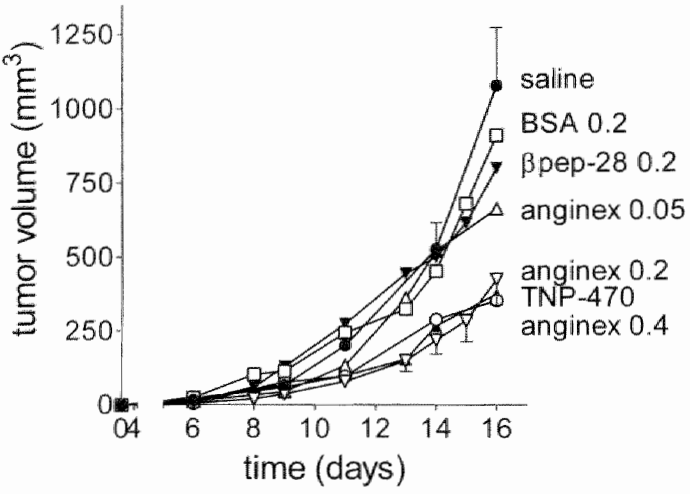

Figure 4.5 *Anginex inhibits tumor growth in mice.*
C57BL/6 mice with B16F10 melanoma injected subcutaneously were treated with saline (n=11, solid circle), 0.05 (n=5, open triangle up), 0.2 (n=14, open triangle down) or 0.4 (n=5, solid triangle up) mg/day anginex or with 0.2 mg/day BSA (n=5, open square) or βpep-28 (n=5, solid triangle down) delivered by osmotic minipumps or with 2 mg TNP-470 every 2 days as of day 9 (n=9, open circle). For clarity variations in tumor measurements (SE was typically roughly 20% in all groups) are only shown for the anginex 0.2 mg/day and the saline groups. Statistical significant inhibition of tumor growth (two way ANOVA test) was observed for anginex doses of 0.2 ($p<0.0001$) and 0.4 mg/day ($p<0.003$) and with TNP-470 ($p<0.0001$).

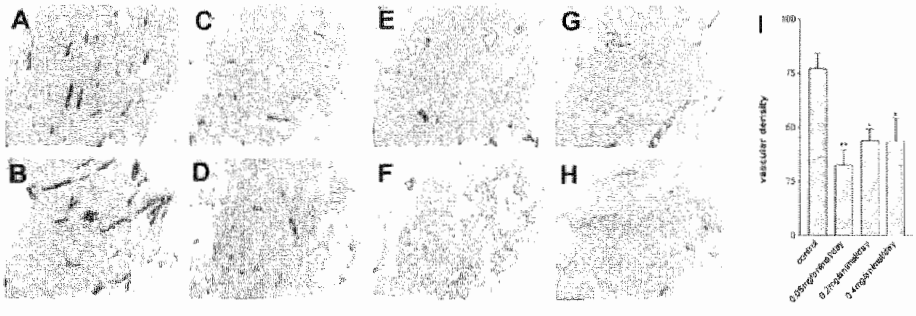

Figure 4.6 *Anginex inhibits angiogenesis in B16F10 melanoma tumors.*
Tumors from mice treated with saline (A,B) or 0.05 mg/day (C,D), 0.2 mg/day (E,F) or 0.4 mg/day (G,H) of anginex were frozen, and 5 μm sections were stained with rat anti-mouse CD31 and peroxidase labeled goat anti-rabbit Ig antibody. Microvessel density (I) was quantified for all tumors as mean number of vessels per 100 mm^2 (±SE, *$p<0.05$, **$p<0.005$).

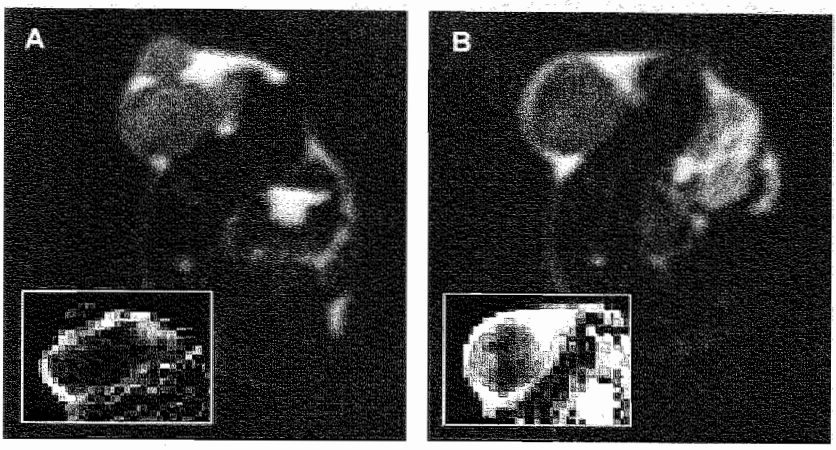

Figure 4.7 *Anginex treatment reduces signal intensity increase in the tumor measured by MRI.* T_2-weighted images illustrate equally sized tumors of an anginex treated animal (A) and a tumor of a BSA treated control animal (B). Subtractions of pre- and post-contrast T_1-weighted tumor measurements of anginex and BSA treated animals are shown in the inserts.

Magnetic resonance imaging (MRI) was used to investigate anatomical features of these mice. Full body cross-sections of T_2-weighted acquisitions are shown in Figure 4.7A and 4.7B. T_1-weighted measurements, given as inserts in Figure 4.7, indicate a smaller increase in signal intensity induced by the contrast agent, in the tumors of anginex-treated animals compared to the signal intensity in tumors of control animals. For anginex-treated animals, the increase in signal intensity over the entire area of the tumor was approximately 50% lower (12.7% ± 3.9%, mean ± SE) compared to that in control animals (26.5% ± 3.6%). Because the signal intensity did not differ significantly at the center of the tumor (anginex 12.5% ± 4.7%, control 17.0% ± 4.5%), this overall difference was the result of a significant reduction (46%) in signal intensity at the rim of the tumor (anginex 17.3% ± 4.3%, control 32.0% ± 3.4%, p=0.04).

Discussion

The aim of the present study, which was to assess efficacy of anginex at inhibiting angiogenesis and tumor growth in mice, has been met. Anginex effectively inhibits both bFGF-induced and tumor-induced angiogenesis, and is capable of inhibiting tumor growth *in vivo* in a mouse model. *In vitro* studies suggest that the mechanism of action for anginex as an angiostatic agent is via apoptosis induction triggered by the inhibition of migration and adhesion of angiogenically activated ECs.

A number of *in vivo* angiogenesis models are available to test new compounds for anti-angiogenic or angiogenic activity (elegantly reviewed in Jain et al.[19]). The

first model chosen here was the chick embryo CAM assay, which is an affordable and straightforward model that is ideally suited for initial screening of potential angiostatic compounds. In this assay, anginex effectively inhibited angiogenesis in a dose dependent manner. The major drawback of the CAM assay in oncological research, however, is that it is a model for developmental angiogenesis, which may not reflect the processes involved in tumor angiogenesis. For this reason, we also used the matrigel plug assay in which angiogenesis is quantified as ex vivo microvessel density determined by immunohistochemistry. The advantage of this method is that the activity of a single angiogenic factor can be studied in a tumor free environment. In the matrigel plug assay, anginex was found to potently inhibit bFGF-induced attraction of blood vessels. Since these angiogenesis model systems indicated *in vivo* inhibition of angiogenesis by anginex, a syngenic melanoma tumor growth model in mice was used to test both anti-angiogenic and anti-tumor activities. For this, we used the rapidly growing B16F10 melanoma model in C57BL/6 mice. In this model, visible tumor mass is present at day 7-9 and the tumor reaches maximal size after 16 days. Anginex demonstrated a profound anti-tumor effect. Although investigation of the anti-tumor mechanisms and the role of anginex in tumor biology was beyond the scope of the present study, its anti-tumor activity is most likely mediated by inhibition of angiogenesis because microvessel density values in anginex-treated tumors were significantly decreased. Results from the MRI experiment showed a reduced signal intensity in tumors of anginex treated animals compared to tumors in control animals. Areas of high signal intensity increase are known to have high microvessel density and vascular permeability[20] and are therefore considered areas with high angiogenic activity. Therefore, these MRI results also support the conclusion that anginex inhibits tumor growth by inhibiting of angiogenesis. Furthermore they lend weight to the concept of using MRI as a surrogate marker for the effects of anginex and perhaps other angiogenesis inhibitors in patients.

In vitro support for the angiostatic activity of anginex is provided by its ability to inhibit EC proliferation, induce apoptosis and inhibit migration in angiogenically activated mouse endothelial cells. The effect of anginex on migration was seen at concentrations that were not inhibitory for proliferation of ECs, indicating that adhesion and/or migration of ECs are specifically inhibited by mechanisms resulting in modulation of proliferation and apoptosis. The results obtained from freshly isolated ECs suggest a preference for activated ECs to undergo apoptosis. While bFGF acted as a survival factor, anginex treatment in the presence of bFGF forced equivalent numbers of ECs into apoptosis as compared to treatment with anginex in the absence of bFGF.

In a recent publication demonstrating the anti-angiogenic activity of anginex, we showed results from human and bovine ECs[15]. Results presented here, indicate a similar response between mouse and human ECs. Any differences can be explained by considering inequivalences in cell growth on one hand, and migratory responses on the other. While activated human ECs are more sensitive to anginex at the cell growth level, mouse ECs are more sensitive to anginex at the level of migration. This difference is a minor consequence in terms of using the mouse

model as an ideal tool for further development of anginex and translational research to the clinic.

These results indicate that anginex is a powerful anti-tumor angiogenesis inhibitor and shows promise to be developed for the treatment of human cancers. Even though this paper only reports on the anti-tumor activity of anginex, the possible broader use of the peptide in the treatment of other diseases, like rheumatoid arthritis, endometriosis, atherosclerosis, psoriasis and ocular neovascularization, requires further investigation and will be the subject of future research in our laboratories.

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Chapter 5

Tumor growth inhibition by and cell biology of anginex

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Abstract

Recently, we described anginex, a *de novo* designed cytokine like peptide that inhibits both *in vitro* and *in vivo* angiogenesis[1]. Here we demonstrate that anginex inhibits tumor growth *in vivo* in an Ls174T human colon cancer model and a B16F10 mouse melanoma model, by inhibition of angiogenesis. Mechanistical studies were performed in *in vitro* and *in vivo* models. The specific binding of anginex to the membrane of endothelial cells (ECs) and its internalization in time by way of receptor mediated endocytosis is demonstrated by flow cytometrical, immunohistochemical, and electron microscopic analysis. Anginex induced detachment and subsequent apoptosis in ECs. The latter was demonstrated using the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD.fmk), which prevented apoptosis induction but still allowed detachment from the matrix. The influence of anginex on cell cycle regulating molecules, like p21, p53 and pRb confirmed apoptosis inducing capacity. Anginex inhibited tube formation of endothelial cells (ECs) in a novel *in vitro* angiogenesis assay.

Introduction

Angiogenesis is crucial for the outgrowth of solid tumors and enhances metastasis formation. The treatment of cancer by inhibiting blood vessel formation is therefore of great promise in cancer treatment. Since most anti-angiogenesis agents identified to date are endogenous molecules and primarily proteins, a different approach has been used in our laboratories. We used rational peptide design which turned out to be a powerful tool in the development of molecules with specific biological activities. Peptides were designed by using a combination approach employing basic folding principles and incorporating short sequences from the β-sheet domains of platelet factor 4 (PF4), interleukin-8 (IL-8) and BPI[2]. Initially, the β-sheet domains from α-chemokines PF4 and IL-8 were used in part to create a presentation scaffold whose solubility in aqueous solution and structural stability were significantly enhanced using basic folding principles[2]. A survey of amino acid sequences from anti-angiogenic proteins revealed that they are compositionally similar, containing numerous hydrophobic and cationic residues. Comparison of the three-dimensional structures of several anti-angiogenic proteins, e.g. endostatin[3], PF4[4], tumor necrosis factor (TNF)[5]and BPI[6], provides a higher level of structural commonality in that they are comprised primarily of anti-parallel β-sheet structure. The selection of novel anti-angiogenic proteins or peptides may be guided by considering all these points: composition high in hydrophobic and cationic residues, anti-parallel β-sheet structure and bactericidal activity. In this regard, βpep peptides, which are designed amphipathic β-sheet-forming peptide 33mers[7;8;9] with bactericidal activity, are obvious candidates.

With this hypothesis, it was found that some of these peptides were able to inhibit endothelial cell proliferation. From a series of 30 βpep peptides, anginex was identified as the most potent inhibitor of angiogenesis[10].

The present chapter describes the anti-tumor activity of anginex in mice, which is mediated by inhibition of angiogenesis. Mechanistic studies demonstrate that anginex is able to induce anoikis in angiogenically activated EC which forces them to undergo apoptosis.

Materials and methods

Cell culture

Human umbilical cord vein derived endothelial cells (HUVEC) were isolated from the vein of human umbilical cords. HUVEC were cultured in culture medium (RPMI 1640, 20% heat inactivated human pooled serum (HS), 2 mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin) on 0.2% gelatin coated culture

flasks. The cells were grown until confluence was reached and sub cultured 1:3 in 0.2% gelatin coated culture flasks or used in experiments (passages 2 to 4).

Antibody preparation

BALB/C mice (2-6 months) were obtained from Charles River Breeding Laboratories (Heidelberg, Germany), maintained on standard laboratory diet and allowed free access to water. Guidelines of the Committee for Care and Use of Laboratory Animals from the Maastricht University were followed throughout. Mice were immunized with 50 µg anginex in distilled water. For the first two immunizations, the anginex solution was emulsified with an equal volume of Specoll. Each mouse received 0.5 ml intra-peritoneally. This treatment was repeated every two weeks. Ten days after the third immunization by the use of an orbital puncture a blood sample was taken and the serum was tested by ELISA for binding to anginex, the lack of binding to BSA. After a high titre of anti-anginex antibodies was detected in the serum, mice were immunized again with the peptide. Five days after the last immunization the mice were sacrificed and the spleen cells were fused with the non-secreting cell line SP_20. After fusion cells were seeded into 96-wells culture plates, containing BALB/C mouse peritoneal macrophages in culture medium consisting of RPMI 1640 (Gibco, Life technologies) with 20% FCS (Life Technologies), $5x10^{-4}$ M β-mercaptoethanol (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and HAT (Life Technologies, Paisley, Scotland), and incubated at 37°C with 5% CO_2. The supernatants were screened for binding to anginex. Positively testing clones were sub cloned by limiting dilution in 96-well culture plates. The antibodies were designated monoclonal after at least three limited dilutions, which showed 100% of the clones positive for the antigen. Two suitable clones, 2D10 and 1B8, of isotype IgG1and IgG3 respectively, were cultured further. The monoclonal antibodies were purified with protein G and protein A chromatography, respectively. The elution fraction was dialyzed to PBS and stored at 4°C.

The rabbit polyclonal antibody was prepared by immunisating a rabbit 4 times over a period of 34 days (at day 0, day 10, day 20 and day 30) with 400 µg anginex. Serum was collected at day 34 and Ig was purified on a protein A column (Pharmingen).

Flow cytometric analysis of anginex binding to HUVEC

HUVEC were either fixed directly with 1% paraformaldehyde (Merck, Darmstadt, Germany) after isolation from the umbilical vein or cultured in a tissue culture flask coated with 1 mg/ml fibronectin in culture medium with or without bFGF for three days. The cells were subsequently detached using 0.125% (w/v) trypsin, fixed in 1% paraformaldehyde at 20°C for 30 minutes and washed twice with washing buffer (PBS / 0.1% (w/v) BSA / 0.01% sodium azide). The cells were incubated for two hours with 25 µM anginex or biotynilated anginex (100 µl per 50,000 cells) at 0°C. After this incubation period cells were washed twice with washing buffer and incubated for one hour with monoclonal antibody (2D10) or

rabbit polyclonal serum directed to anginex at a concentration of 5 µg/ml followed by a one hour incubation with goat anti-mouse Ig-FITC (DAKO, Denmark), goat anti-rabbit Ig-FITC (DAKO) or streptavidin-PE (DAKO). After this hour cells were washed once and the FL-1 H or FL-2 H fluorescence was analyzed on a FACScalibur (Beckton Dickinson). 5,000 cells were recorded for each sample and the experiment was repeated 3 times with different HUVEC isolations.

Immunohistochemical staining of anginex on endothelial cells

HUVEC were cultured under normal conditions or in the presence of 25 µM anginex for 3 days. After trypsinization the cells were diluted in 10% normal bovine serum and cytospinned on glass slides. The cytospin preparations were dried overnight and fixated in methanol/H_2O_2 (50:1 dilution). After washing the slides twice in PBS they were blocked in 5% BSA in PBS for 15 minutes. The slides were washed twice and incubated in 5 µg/ml Rabbit anti anginex polyclonal antibody for one hour and then washed 3 times in PBS. The second antibody was biotinylated goat anti-rabbit IgG diluted 1 in 200 and after washing again 3 times the slides were incubated in avidin-biotin-peroxidase complex. The enzymatic reaction was visualized with DAB and H_2O_2 and the slides were counterstained with hematoxillin.

Electron microscopic analysis of binding of anginex to endothelial cells

Sterile thermanox cover slips were coated with 1% gelatin at 37°C for 30 minutes. After that the cover slips were rinsed once with PBS and 1 ml 0.5% glutardialdehyde was added and incubated for 30 minutes to fixate the gelatin. The thermanox cover slips were subsequently washed twice with PBS and twice with culture medium. After this 10,000 cells (HUVEC) in a volume of 1 ml were placed in a 24-wells well containing the coated cover slips. After one hour of culture the medium was replaced by medium containing 75 µM anginex and cells were grown for either 2 or 24 hours. The HUVEC were prepared for ultra structural investigation by fixating the cells on the thermanox with 1% paraformaldehyde and localization of anginex by jet freezing and freeze substitution, as described previously[11]. The anginex was detected by staining with a polyclonal rabbit IgG antibody against anginex followed by a one hour incubation with an immunogold labeled goat anti-rabbit Ig antibody (Aurion, Wageningen).

Analysis of apoptosis induction on the flow cytometer

HUVEC were cultured in the presence or absence of 25 µM anginex with or without ZVAD-fmk or with or without pre-incubation for 3 days with thymidine. After 3 days of culture cells were detached with trypsin. The cells were spun down at 1500 rpm for 5 minutes and the pellet was resuspended in 70% ethanol and incubated for two hours at -20°C. Subsequently cells were centrifuged at 1500 rpm for 5 minutes and the cell pellet was resuspended in 200 µl DNA extraction buffer (90 volumes 0.05 M $Na_2HPO_4.2H_2O$, 10 volumes 0.025 M citric acid, 1 volume 100% triton-X100, pH 7.4) and incubated for 20 minutes at 37°C. After the

incubation period propidium iodide (PI) was added to a final concentration of 20 μg/ml and the DNA profile was directly analyzed on the FACScalibur.

Terminal deoxynucleotidyl transferase (TdT)- mediated digoxigenin-dUTP nick end labeling (TUNEL) analysis of apoptosis

Cytospin preparations were made from HUVEC cultured under diverse conditions as described previously. In short HUVEC were harvested by trypsin treatment and spun down for 5 minutes at 1500 rpm. Cells were subsequently washed once with PBS and resuspended in PBS/10% normal bovine serum and spun on the slides at a speed of 500 rpm. The slides were dried and endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. Sections were pre-treated with 20 μg/ml of protein-digesting enzyme for 15 minutes at room temperature. After washing in 4 changes of aquadest, equilibration buffer (Apoptag kit, Intergen) was applied for 10 minutes at room temperature, followed by the application of 10 Tl working strength TdT enzyme (a mixture of 7.6 Tl reaction buffer and 3.2 Tl TdT enzyme, Apoptag kit, Intergen) per cm^2 of cells for 1 hour at 37°C in a humidified chamber and covered with a cover slip. The reaction was terminated in preheated Stop/Wash buffer (Apoptag kit, Intergen) for 30 minutes at 37°C. After washing in PBS, the digoxigenin-labeled dUTP polymer was detected by anti-digoxigenin-peroxidase (Apoptag kit, Intergen) for 30 minutes at room temperature. After washing, visualization was performed with DAB. The color reaction was followed microscopically, and the reaction was terminated when adequate staining was achieved. Sections were counterstained lightly with hematoxylin, and after dehydration they were mounted with entellan. A rat thymus was taken into the reaction as a positive control, and the TdT enzyme in the working-strength TdT was replaced by aquadest as a negative control. For the evaluation of TUNEL staining, light microscopic evaluation was performed at a magnification of × 400 with the aid of a counting grid. TUNEL-positive nuclei were counted in 10 microscopic fields to a total of 500 HUVEC.

Role of p53 and bcl-2 in apoptosis induced by anginex

HUVEC were cultured on a gelatin coat and synchronized in a quiescent state (G_0) by starvation (RPMI-1640 medium supplemented with 1% horse serum, 0.25% BSA) for 20 to 24 hours and were then stimulated with either epithelial growth factor (EGF) (20 ng/ml) alone or EGF (20 ng/ml) and anginex (100 μg/ml) or βpep-28 (100 μg/ml) in RPMI-1640 medium supplemented with 2.5% fetal bovine serum (FCS). To prepare cdk2 immunoprecipitates, 6×10^6 cells, washed twice with ice cold buffered saline (PBS), were lysed in 0.5 ml of "lysis buffer" (0.05 M Tris pH 8.0, 0.150 M NaCl, 1% Triton X-100, 0.1 M NaF, 0.0025 M EGTA, 0.001 M EDTA, 0.0014 M phenylmethylsulfonyl fluoride, 0.001 M $NaVO_4$, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.2 μg/ml okadaic acid). Protein concentration was determined by the bicinchoninic acid (BCA) assay. Four hundred micrograms of total protein lysate diluted in "immunoprecipitation buffer" (lysis buffer without okadaic acid) to a final volume of 1 ml was incubated for 1

hour on ice with 50 µl of a 50% suspension of agarose beads conjugated with nonimmune rabbit IgG (preclearing), followed by centrifugation to remove beads. The supernatant was incubated for 1 more hour on ice with 50 µl of a 50% suspension of agarose beads conjugated with rabbit IgG anti-human cdk2. To reduce non-specific binding, the beads were preincubated for 1 hour in immunoprecipitation buffer, adsorbed proteins were dissociated by boiling in 1× SDS-sample loading buffer (0.05 M Tris/HCl pH6.8, 10% glycerol, 1% SDS, 0.02% bromphenol blue, and 5% β-mercaptoethanol), fractionated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), and electroblotted (0.01 M CAPS, 10% methanol, pH 11) onto a PVDF membrane. After blocking of non-specific binding (blocking buffer:0.02 M Tris pH 7.8, 0.145 M NaCl, 0.1% Tween-20, 20% FBS), the membrane was immunoblotted (0.02 M Tris pH 7.8, 0.145 M NaCl, 0.05% Tween-20) with the indicated antibodies. Secondary antibodies (horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG) were visualized using the Super Signal Ultra or ECL chemiluminescent substrates. Immunoblotting of total protein was performed as described previously, but only 50 µg of total protein diluted in 2 × SDS-sample loading buffer was loaded per lane. Discontinuous 12.5% SDS-PAGE was used in all cases except for pRb analysis, where a 5% to 8.5% SDS-PAGE gradient gel was chosen. Immunoprecipitation and immunoblot analyses were performed for each of three (or more) independent, but identical experiments and demonstrated reproducibility of results [12].

Measurement of caspase-3 activation

HUVEC were cultured for 3 days in the presence or absence of 100 µg/ml of anginex. After this culture 10×10^6 endothelial cells and also a sample of Jurkatt cells (control) were harvested with 0.5% EDTA and sonicated. The samples were spun down and supernatant was collected for analysis of caspase-3 activation using a fluorometric immunosorbent enzyme assay (Roche). In short a microplate was coated with anti-caspase-3 antibody, unspecific binding sites were blocked and lysates from cells were added. After washing Ac-DEVD-AFC was added. In the presence of caspase the substrate was cleaved into the fluorescent AFC, which was fluorometrically detected.

In vitro angiogenesis assay

An *in vitro* angiogenesis model was used to test different concentrations of anginex as described before[13]. In short endothelial cells were grown on gelatin coated cytodex-3 beads. The beads were subsequently embedded in a collagen gel (vitrogen-100, Collagen Corporation, California, USA) and sprout formation was stimulated with bFGF (Sanvertech) on top of the gel in the medium. The substances to be tested were also placed in the medium on top of the gel.

Ls174T tumor model

Nu/nu swiss mice (Charles River, Heidelberg, Germany) of 6 weeks old were inoculated on the right flank with 10^6 Ls174T colon cancer cells. After tumors had grown to approximately 50 mm^3, mice were treated for 14 days with intra-tumoral injections of 35 mg/kg/day BSA, 35 mg/kg/day anginex or 35 mg/kg/day βpep-28 in a volume of 100 µl. Tumor size was determined by measurement of the width and length of the tumors and calculating the volume with the formula $width^2$ times length times 0.52. After the 14 day treatment animals were sacrificed and vessel density stainings were performed. Frozen tissue sections were fixed in acetone for 10 minutes, air dried, pre-treated with PBS/ 0.1% BSA/ 1% human serum and subsequently stained for 1 hour with a phycoerythrin-conjugated monoclonal antibody against CD31 (Pharmingen, San Diego, CA). Sections were evaluated by fluorescence microscopy and analyzed by Metamorph software. Standardized quantitative analysis of pixel density was performed using Scion-image software.

B16F10 melanoma model

C57BL/6 inbred mice (Charles River, Heidelberg, Germany) were 6 weeks of age at the start of the experiment. At day 0 200,000 B16F10 cells (a kind gift from Dr J. Fidler) were inoculated subcutaneously on the right flank. On the left flank an Alzet mini osmotic pump (Durect) with a pumping time of minimally 14 days was placed on the same day. The Alzet pumps contained either 0.9% NaCl or 14, 7 or 1.75 mg/kg/day anginex or as controls βpep-28 or BSA at 14 mg/kg/day. Between day 6 and 9 the tumors were visible and were thereafter measured daily. The volume was calculated as $width^2 \times length \times 0.52$. On day 16 the mice were sacrificed and the tumor was fixed in liquid nitrogen. 5 µm thick sections were made of the tumors and these were stained with rat anti-mouse CD31 antibody and peroxydase labeled goat anti-rat Ig (Immunotech, France).

Results

Anginex inhibits tumor growth in mice

Anginex was tested for anti-tumor activity in the Ls174T human colon carcinoma xenograft model. After tumors had grown to approximately 50 mm^3, mice were treated for 14 days with anginex, βpep-28 or BSA (35 mg/kg/day). Due to the fact that rapid renal excretion was expected (MW 3.8 kD), treatment was performed by loco-regional s.c. injection at the tumor site. After two weeks of treatment, control animals developed tumors of approximately 1500 mm^3. In the mice treated with anginex, 50% of the animals did not develop any tumor mass, resulting in an overall 30-50% inhibition of tumor growth relative to the tumors in the control mice treated with either βpep-28 or BSA (Figure 5.1A-C). After the two

weeks treatment, mice were sacrificed and the tumors were excised for immunohistochemical staining of blood vessels using CD31 antibodies to analyze anti-angiogenic effect of anginex. An 80% inhibition of microvessel density was observed in anginex treated animals (Figure 5.1E) compared to saline treated control animals (Figure 5.1D).

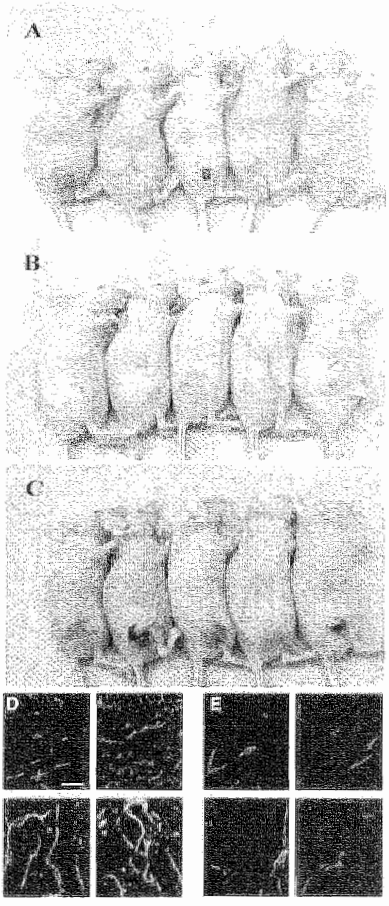

Figure 5.1 *Anginex inhibits tumor growth in Ls174T inoculated mice.*
Mice were treated with PBS (A), anginex 35 mg/kg/day (B) or βpep-28 35 mg/kg/day (C) and vessel staining was done on tumors from the control animal (D) and from the anginex treated animals (E) after resection of the tumor with a PE conjugated CD31 antibody.

Due to the formation of a white precipitate at the injection site (which resolved over a period of 1-2 days), it was difficult in these experiments to measure the exact tumor size. Because of this, and because of the fact that the Ls174T tumors grew rapidly to large tumors with massive central necrosis, another tumor model was chosen to exactly quantify the anti-tumor effects of anginex. C57BL/6 mice, inoculated with B16F10 mouse melanoma cells, were treated with saline (0.9%

NaCl), control peptide βpep-28 at 7 mg/kg/day, BSA at 7 mg/kg/day or with 1.75, 7 or 14 mg/kg/day anginex delivered by an osmotic pump, which was placed in the s.c. space of the left flank. These experiments were performed as a prevention model by inoculation of the tumor and implantation of the pump at the same time. Saline treated animals developed a palpable tumor mass at day 7 growing to 1000 mm^3 on day 16. A concentration dependent inhibition of tumor growth was observed by treatment with anginex of 59% at 7 mg/kg/day (Figure 5.2A). Tumor sections of control treated and anginex treated animals were stained with CD31 antibody. Vessel density was downregulated 43% by treatment of animals with 7 mg/kg/day anginex (Figure 5.2B).

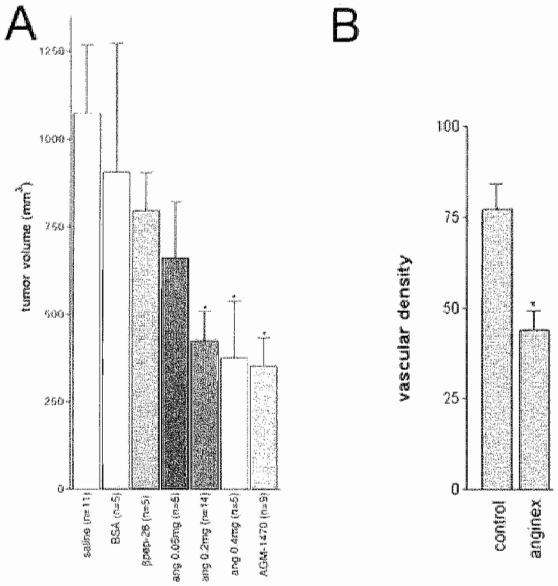

Figure 5.2 *Anginex inhibits tumor growth in B16F10 melanoma inoculated mice.*
Mice were treated with 0.9 % NaCl, 7 mg/kg/day BSA, 7 mg/kg/day βpep-28 or with 1.75, 7 or 14 mg/kg/day anginex by continuous administration via osmotic mini pumps and tumor volume was measured on day 16 after inoculation (A). After resection of the tumors sections were made of the saline control tumors and tumors from animals treated with 7 mg/kg/day anginex. The sections were stained with anti-CD31 antibody and peroxidase conjugated goat anti-rat Ig , after which vessel density was determined (B).

Anginex inhibits in vitro angiogenesis

The above-described results demonstrate that anginex inhibits angiogenesis *in vivo*. To investigate the mechanism by which anginex inhibits angiogenesis the *in vitro* activity of anginex on endothelial cells was tested. The *in vitro* angiostatic capacity of anginex was demonstrated in the recently described *in vitro* angiogenesis/tube formation assay. Anginex was able to inhibit the bFGF-induced sprout formation of bovine capillary ECs (BCE) by 74% at 25 μM (Figure 5.3A

and B). The inhibition was concentration dependent and was half-maximal at a concentration of 0.25 μM (Figure 5.3C). PF4 was used as a positive control and inhibited angiogenesis as strong as anginex.

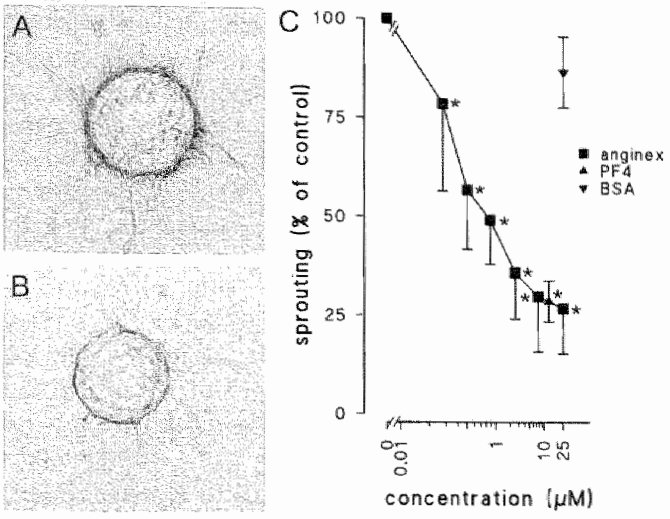

Figure 5.3 *Anginex inhibits in vitro angiogenesis induced by bFGF.*
BCE grown on a gelatin coated bead are induced to form sprouts by bFGF (A), when anginex is added on top of the gel the sprout formation is inhibited (B). The inhibitory effect of anginex on sprout formation is concentration dependent (C, mean ± SEM, *$p<0.02$).

Anginex directly binds to activated ECs

As a tool for studies on the mechanism by which anginex binds endothelial cells, a polyclonal rabbit antiserum and the mouse monoclonal antibody 2D10 were prepared. Using overlapping dodecapeptides the binding epitopes of the antibodies were mapped by ELISA as shown in Figure 5.4. While both the monoclonal and the polyclonal antibodies were found to recognize the C-terminal part of anginex, the polyclonal antibody also recognize the N-terminal part of anginex with a 3 times lower affinity (data for ELISA are not shown).

In order to elucidate whether the anti-angiogenic effects of anginex are caused by direct binding to ECs, binding of anginex to endothelial cells was examined by using flow cytometry, immunohistochemistry and electron microscopy. Anginex binds to human umbilical vein derived endothelial cells (HUVEC) as indicated by fluorescence measurement of (i) ECs stained with fluorochrome labeled anginex or (ii) indirect detection of bound anginex using 2D10 anti-anginex antibody and FITC-conjugated goat anti-mouse Ig antibody (Figure 5.5A). In an attempt to investigate intracellular trafficking of anginex, HUVEC were cultured with anginex for three days and subsequently cells were harvested and cytospin preparations were made. Staining of the cytospins with the polyclonal rabbit anti-anginex antibody yielded the presence of anginex on the cell membrane and in

vesicles inside the cells (Figure 5.5 B). Electron microscopic analysis of the binding of anginex to HUVEC under culture conditions indicated that the peptide was internalized time dependently into the cells by receptor mediated uptake and endocytosis (Figure 5.5 D). Also it was observed that culture with anginex after 24 hours resulted in peptide accumulation at the 'abluminal' or matrix-binding site of the ECs.

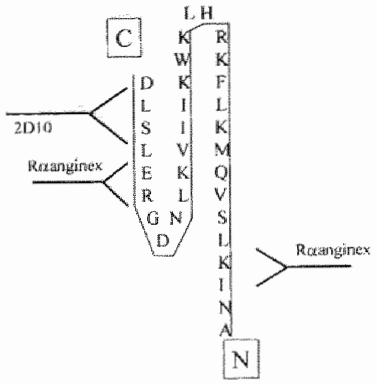

Figure 5.4 *Antibody 2D10 binds the C terminus, whereas the Rabbit polyclonal antiserum binds both the C and N terminus of anginex.*

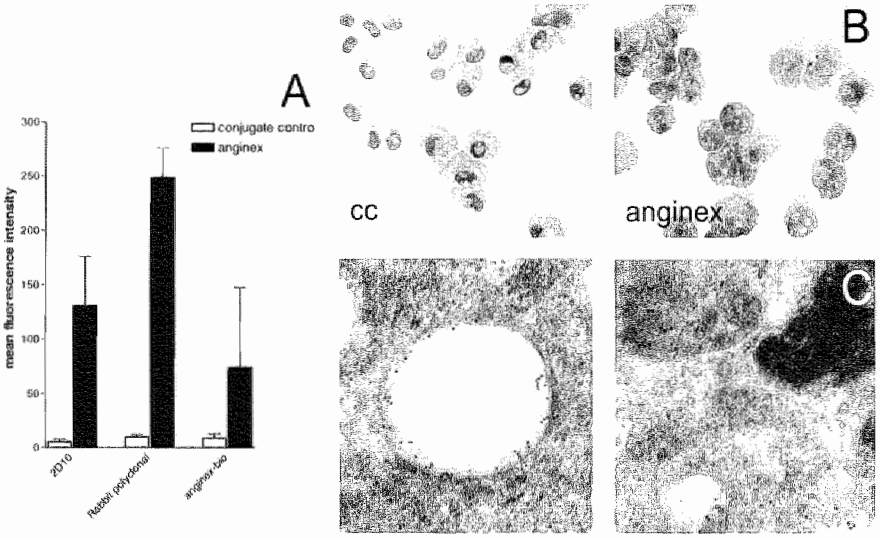

Figure 5.5 *Anginex binds to endothelial cells.*
HUVEC were stained with anginex and 2D10 or polyclonal rabbit serum and fluorescent conjugated or with biotinylated anginex and streptavidin-phycoerythrin as described in the materials and methods section. The first bar of each condition is the conjugate control and the second bars are the anginex stained HUVEC (A). Immunohistochemical staining shows anginex staining on the membranes of HUVEC and inside vesicles (B). Electron microscopic analysis more clearly shows a membrane staining inside vesicles in the HUVEC (C). Black dots in the pictures are immunogold labelled staining for anginex.

For angiogenesis inhibition to be used as a therapy (in the treatment of cancer) it is important that only the angiogenically stimulated and not the quiescent vessels are affected. Two approaches were used to study the binding and efficacy of anginex on resting as well as actively dividing endothelial cells were investigated. Firstly, binding of anginex to endothelial cells in several growth phases was examined. Endothelial cells were either (i) directly fixed after isolation from the umbilical vein, (ii) grown for three days in normal culture medium or (iii) grown for three days in the presence of 10 ng/ml bFGF. The cells were subsequently fixed in paraformaldehyde and incubated with anginex, prior to indirect staining with 2D10 anti-anginex antibody. A gradual increase in peptide binding was observed with increasing activational state (Figure 5.6A).

Because binding of anginex to ECs might not be informative of its efficacy, the second approach made use of the ability of anginex to induce apoptosis (see below) in actively growing in ECs. HUVEC were either stimulated with bFGF or stimulated with bFGF in the presence of excess thymidine in order to halt cell cycle in, or at, S-phase. Although the latter procedure may not decrease the metabolic state of the cells, it resulted in a significant (approx. 50%) reduction of anginex induced apoptosis (Figure 5.6B).

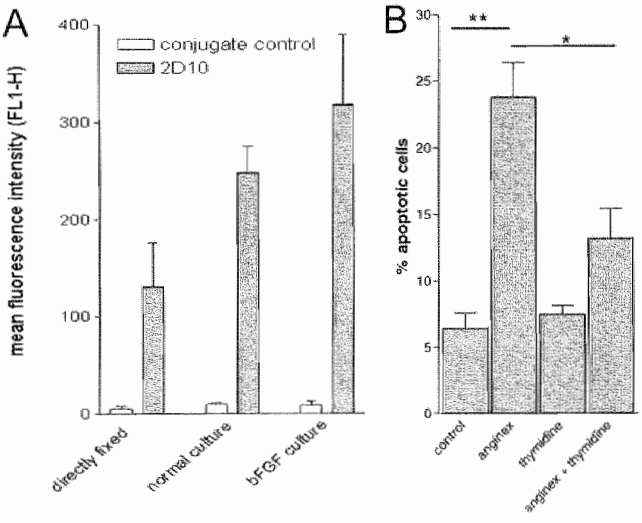

Figure 5.6 *Anginex binds and affects activated endothelial cells.*
HUVEC cultured for three days in serum rich or serum rich medium supplemented with 10 ng/ml bFGF bind more anginex than HUVEC directly fixed after isolation from the umbilical cord vein (A). When HUVEC are preincubated for three days with thymidine the cells are stopped in cell cycle and anginex is unable to induce apoptosis in these cells (B).

Anginex induces apoptosis in endothelial cells

From an earlier study it was suggested that anginex induced apoptosis in activated ECs, whereas the control peptide βpep-28 did not induce apoptosis (Figure 5.7A). Apoptosis induction was confirmed by TUNEL analysis (data not shown) and by measurement of caspase-3 activity, which was approximately 4 times higher when HUVEC were cultured in the presence of 25 μM anginex for 3 days (data not shown). To confirm the involvement of caspase activity, HUVEC were cultured in the presence or absence of 25 μM anginex and in the presence or absence of 100 μM ZVAD, a specific caspase 3 inhibitor. ZVAD blocked the apoptosis induction induced by anginex, measured by subdiploid peak analysis in the flow cytometer (Figure 5.7B), however cells in culture still detached from their matrix (not shown). This indicates that apoptosis induction by anginex is mediated by inhibition of adhesion.

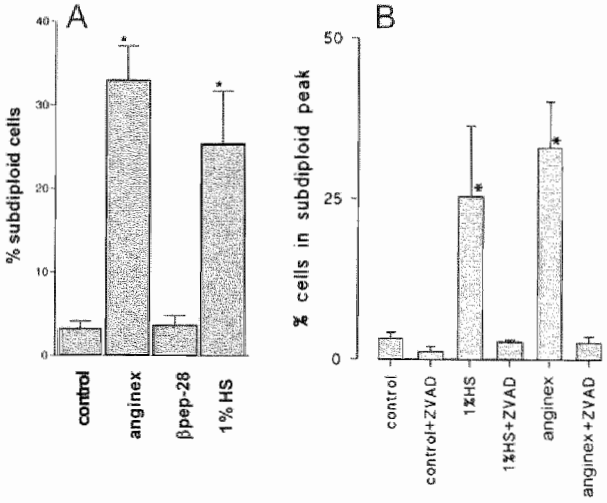

Figure 5.7 *Anginex induces apoptosis.*
(A) % of subdiploid cells in a control HUVEC culture, or HUVEC cultured in the presence of 25 μM anginex or βpep-28 (peptide which is similar to anginex, but shows no anti-angiogenic activity) after 72 hours of culture. (B) ZVAD-fmk was added to HUVEC cultures in the presence or absence of 25 μM anginex, where after subdiploid cells were analysed on the FACScalibur. As a positive control HUVEC were starved by culturing in only 1% HS.

Role of anginex in controlling the cell cycle

PF4 is known to negatively regulate the cell cycle. It induces an accumulation of endothelial cells in S-phase of the cell cycle[14]. For the progression into the G1 phase of the cell cycle, phosphorylation of the retinoblastoma protein (pRb) is necessary. PF4 has been shown to attenuate the up regulation of phosphorylation pRb (ppRb)[12]. This also occurred when ECs were treated with anginex (Figure 5.8E). PF4 also has effects on $p21^{Cip1/WAF1}$ and its complex formation with cdk2. It

prevents down regulation of $p21^{Cip1/WAF1}$ by EGF and up regulates the complex formation between $p21^{Cip1/WAF1}$ and cdk2 in EGF stimulated HUVEC[12]. Anginex had similar effects on $p21^{Cip1/WAF1}$ and the $p21^{Cip1/WAF1}$/cdk2 complex in EGF stimulated endothelial cells (Figure 5.8B, C). The amount of cyclin E associated with cdk2 which is necessary for pRb phosphorylation was not affected by anginex (Figure 5.8D), which also had been found for PF4[12]. The expression of p53 showed to be somewhat down regulated by EGF, which was inhibited by anginex (Figure 5.8A). For PF4 these regulations result in attenuation of the cell cycle and for anginex even in apoptosis induction of endothelial cells.

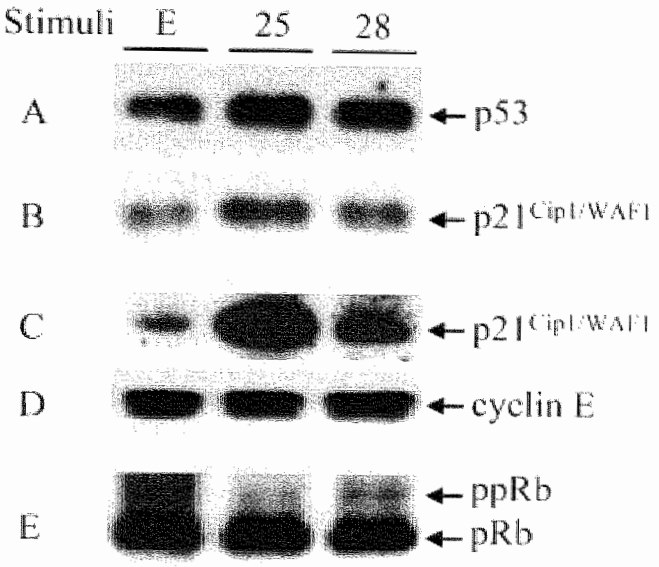

Figure 5.8 *Anginex influences the cell cycle.*
HUVEC were stimulated with EGF and incubated in RPMI-1640, supplemented with 2.5% FCS (top E), in the presence of anginex (25), or in the presence of βpep-28 (28) for a period of 15 hours. The expression of p53 (A), $p21^{Cip1/WAF1}$ (B), $p21^{Cip1/WAF1}$/cdk2 complex (C), cyclin E/cdk2 complex (D), pRb and ppRb (E) were examined by immunoprecipitation and Western Blot analysis.

Discussion

Most anti-angiogenic drugs only have one effect on the angiogenic process. These effects can be influencing the angiogenic factors released by a tumor, interference with endothelial cell growth, inhibition of the migration of endothelial cells in the direction of the angiogenic stimulus (the tumor), or modulation of proteolytic enzymes (matrix metalloproteinases), which are secreted by the endothelial cells and are necessary to make their way free to move through the extracellular matrix. For example PF4 inhibits endothelial cell growth[14], CAI

inhibits proliferation of endothelial cells[15], marimastat influences the action of matrix metalloproteinases[16], and the $\alpha_v\beta_3$ antibodies LM609 and accutin induce apoptosis in endothelial cells by prevention of adhesion of these cells to a matrix[17;18]. Anginex has been developed for two main reasons: (i) to overcome the possibility that endogenous inhibitors have more diverse effects on different tissues, and (ii) to develop a more potent angiogenesis inhibitor, with diverse effects on the angiogenic process.

The purposes of the current study were to see if anginex inhibits tumor growth by inhibition of angiogenesis and to elaborate the way in which it inhibits angiogenesis. In order to investigate anti-tumor effects of anginex two mouse models were used. These two models were both tumor growth prevention models, Ls147T human colon carcinoma and B16F10 mouse melanoma. Anginex was able to inhibit tumor growth as compared to control in both these models, but did not interfere with tumor take. CD31 staining indicated that this tumor growth inhibition was the result of an impaired formation of new blood vessels.

In vitro studies of angiogenesis inhibition by anginex started with testing in a novel *in vitro* model. In this model the sprout formation, which is the result of both endothelial cell growth and migration, was inhibited by anginex. The main focus in the mechanistic studies was elaboration on apoptosis induction by anginex. Binding studies showed that anginex directly bound to endothelial cells and was internalized in time in a membrane bound manner by way of endocytosis. Pinocytosis without membrane binding was also seen. The receptor leading to membrane bound internalization has not been elucidated yet. It is also not clear whether this membrane receptor binding or internalization is needed for the apoptosis induction to occur or whether the apoptosis is induced by stopping the cell cycle.

The receptor for most angiogenesis inhibitors has not yet been identified and also the exact action of the endogenous angiogenesis inhibitor PF4 has not yet been elucidated. However it is known that PF4 is secreted by activated platelets[12] and is a member of the CXC family of chemokines. It does not have any proinflammatory effects and is known to have a high binding affinity for heparin and heparan sulfate and is also known to bind heparan sulfate on the endothelial surface. PF4 has anti-proliferative effects on endothelial cells, by blocking cell cycle entry and progression into S phase[19]. PF4 however does not induce apoptosis in endothelial cells, in contrast to anginex. This implies that there is another mechanism of action for anginex than there is for PF4 to inhibit angiogenesis. The receptor for PF4 on endothelial cells has not been discovered until now, however it is known that the CXC sequence in the molecule plays an important part in its receptor binding. Also IL-8 binds via the CXC sequence to its receptors, however the receptor for IL-8 has not yet conclusively shown to be present on endothelial cells. The CXC sequence present in PF4 and IL-8[20] is not present in anginex. This is probably the explanation for the different effects of the peptide. The ELR motif present in IL-8 is essential for its angiogenic activity. When this sequence is replaced the angiogenic activity is traded for an anti-angiogenic activity[21]. This might also play a role in the anti-angiogenic activity of anginex. Possibly the only corresponce

between anginex and PF4 is the fact that heparin blocks both their action[22]. Though some of the effects anginex has on endothelial cells are quite similar to the effects brought about by PF4 (the effects on the cell cycle proteins), other effects are different. In this respect the peptide probably more closely resembles bactericidal/permeability-increasing protein (BPI), which also induces apoptosis in endothelial cells.

From these studies it can be concluded that the de novo synthesized peptide anginex has anti-tumor activity by inhibition of angiogenesis. It stops cell cycle progression in endothelial cells and even induces apoptosis in these cells. The induction of p53 results in apoptosis on the one side and on the other side p53 upregulates p21 formation. The last subsequently downregulates cyclin-E-cdk2 complex formation, leading to a downregulation of phosphorylated pRb, which eventually results in a downregulation of DNA synthesis. Anginex directly binds to endothelial cells and is internalized in the endothelial cells by receptor-mediated uptake, however whether this binding results in apoptosis induction still has to be shown.

Anginex shows great promise as a novel anti-tumor agent, however more preclinical studies should be done, which will also give more insight into the anti-angiogenic mechanism.

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Chapter 6

The anti-angiogenic properties of bactericidal/permeability-increasing protein (BPI)

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Abstract

Inhibition of angiogenesis is regarded as a promising tool in the treatment of diseases such as cancer, arthritis and atherosclerosis. This fact has lead to the search for novel endogenous or synthetic angiogenesis inhibitors. Recently, anti-angiogenic properties were ascribed to an endogenous molecule that until only recently was known for its antibacterial effects. This molecule, bactericidal/permeability-increasing protein (BPI), that was discovered as a bacterial permeabilizing and LPS neutralizing protein, was found to inhibit angiogenesis by specific induction of apoptosis in endothelial cells. This paper gives a short introduction on angiogenesis and reviews the current knowledge on BPI as an angiogenesis inhibitor. In addition, the issue of commonality between antibacterial and anti-angiogenic functions will be addressed.

Introduction

The process of new blood vessel formation, angiogenesis, is currently a major focus in medicinal science. Next to an important role in the development of normal tissues and physiological processes such as wound healing, angiogenesis is of key importance in the pathology of cancer, rheumatoid arthritis and cardiovascular diseases. Because of the fact that these diseases are dependent on angiogenesis, inhibition of angiogenesis may be a means of treatment of the disease. This urged researchers to discover molecules, either endogenous, exogenous or synthetic, which inhibit angiogenesis *in vivo* and are suitable for the treatment of patients. The most investigated inhibitors along with their activities on angiogenesis are presented in Table 1.1 (see Chapter 1). To date anti-angiogenesis therapy is considered a promising approach, possibly leading to the desperately needed breakthrough in the treatment of the aforementioned diseases. Indeed, preclinical testing of some of the recently discovered angiogenesis inhibitors has been promising and several of them are currently undergoing clinical testing[1].

Table 6.1 BPI induces cell death in BCE, MVEC and HUVEC as measured by trypan blue uptake.

	HUVEC	MVEC	BCE
Control	5*	7	4
BPI	25	22	36
bFGF	5	7	2
bFGF and BPI	30	33	28

Endothelial cells were cultured on a fibronectin coat for three days with or without 10 ng/ml bFGF and in the presence or absence of 1.8 μM BPI. After this culture period the cells were harvested by trypsinisation and stained with trypan blue, the cells with a blue stained nucleus have a permeabilized membrane, which occurs during cell death.
*Data are presented as percentage of trypan blue stained cells

The angiogenesis cascade is an intricately regulated process that occurs as a result of the growth of capillaries by vascular sprouting from pre-existing vessels. Hypoxia is a major inducer of angiogenesis leading to the expression of growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF) and angiopoietins that can activate the quiescent endothelial cells of a pre-existent blood vessel to enter cell cycle. Activated endothelial cells start producing specific proteases (matrix metalloproteinases, MMPs) that degrade the underlying basement membrane and extracellular matrix to facilitate migration in the direction of the stimulus. Subsequently, the endothelial cells also start to proliferate and eventually cells have to differentiate to form a lumen, attract pericytes and smooth muscle cells and secrete a new basement membrane and extracellular matrix. Only when these latter processes of differentiation have occurred, is the vessel considered mature and functional (Figure 1.1, see chapter 1)[2].

Over the last years, more than 280 novel angiogenesis inhibitors have been described, of which a minority also have activity *in vivo*. The most promising are those that inhibit angiogenesis by a direct inhibitory effect on endothelial cell growth. Among them are AGM-1470, angiostatin, endostatin, vasostatin, restin, platelet factor 4 and anginex. The fact that lack of drug-induced resistance and regression of tumors was observed in mouse models was recognized as a major progress. This is best exemplified by the endogenous inhibitor endostatin, which is a proteolytic fragment of collagen XVIII. The list of endogenous proteins with anti-angiogenic activity being identified is continuously growing. Detailed mechanisms of action, however, have not yet been described for most of these angiogenesis inhibitors.

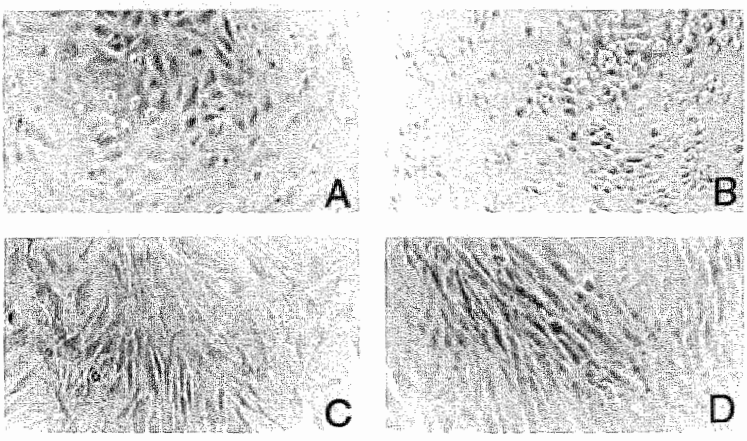

Figure 6.1 *BPI induces anoikis in endothelial cells.*
Human endothelial cells (A, B) and fibroblasts (C, D) were cultured in normal culture medium in the absence (A, C) or in the presence (B, D) of 1.8 μM BPI, for three days. In the presence of BPI only the endothelial cells detach from their underlying matrix.

Excess angiogenesis can be blocked or inhibited in various ways. One way is to modulate endothelial cell growth, which can be achieved by directly binding to and affecting the endothelial cells (described above) or by competition for, or neutralization of, angiogenic factors. These angiogenic factors include VEGF and FGF. The development of tumors is very often dependent on the presence of these factors. Angiogenesis and subsequent tumor growth can, therefore, be inhibited by blocking these factors. There are different ways to achieve this; with the use of humanized blocking antibodies to these factors or antibodies to their receptors, with soluble receptors functioning as antagonists, by using dominant negative growth factor variants, or by the use of growth factor antisense constructs. Functional interference with growth factor signaling can also be performed by specific inhibitors of growth factor receptor signaling, as has been described for SU5416 and SU6668, specific inhibitors of growth factor receptor phosphorylation.

Aside from inhibition of endothelial cell growth, angiogenesis can also be modulated by affecting endothelial cell adhesion to, or migration through,

components of the extracellular matrix. Efforts are also being made to search for modulators of these interactions. The first identified member of this group of compounds is the endogenously produced cytokine interferon-α. Anti-endothelial activity was recognized by the observation that interferon could inhibit the migration of capillary endothelial cells. Subsequently, both interferons-α and -β were shown to have *in vivo* anti-angiogenic activity. The demonstration of over expression of receptors for extracellular matrix components focused on the interaction of endothelial cells with the matrix as a target for inhibition of angiogenesis. This turned out to be a relevant approach by demonstrating that ligation of over expressed $\alpha_v\beta_3$-integrin molecules (the biological function of which is binding of vitronectin and other RGD-containing matrix components) with an antibody called LM609 interferes with endothelial cell growth leading to inhibition of angiogenesis and subsequent tumor growth. The exposure of endothelial cells to anti-$\alpha_v\beta_3$ antibodies results in the induction of apoptosis through the detachment of these cells from the extracellular matrix. Another mechanism of angiogenesis inhibition, which is related to the inhibition of endothelial cell adhesion and migration, is the use of specific inhibitors of proteinases that dissolve the connective tissue. Matrix metalloproteinases are an homologous family of enzymes that are involved in tissue remodeling and morphogenesis. Collectively, these enzymes are capable of degrading all components of the extracellular matrix. Increased activity of these enzymes has been observed during tumor formation, and therefore inhibitors of MMPs represent an attractive approach to treat cancer. MMP inhibitors can be divided in synthetic protease inhibitors and naturally occurring MMP inhibitors, the tissue type inhibitors of metalloproteinases or TIMPs. Belonging to the former group, marimastat and batimastat, potent broad-spectrum inhibitors of the major MMPs, can prevent or reduce the spread and growth of a number of different malignant tumors in numerous animal models. Cell adhesion and proteolytic mechanisms are functionally associated, as recently demonstrated by the observation that the collagenase MMP-2 can bind to integrin $\alpha_v\beta_3$ on angiogenic blood vessels. Most interestingly, it was found that a naturally occurring MMP-2 breakdown product, called PEX, can inhibit cell associated collagenolytic activity. It has been suggested that this breakdown product is an important regulator of protease activity during angiogenesis and vasculogenesis. A recombinant form of PEX was useful in blocking angiogenesis and tumor growth *in vivo*, providing a novel therapeutic approach for angiogenesis inhibition at this level.

The recently discovered angiogenesis inhibitory effect brought about by the bactericidal agent bactericidal/permeability-increasing protein (BPI) will be discussed in the following section.

Bactericidal/permeability-increasing protein (BPI)

Bactericidal/permeability-increasing protein (BPI) is an approximately 55 kD protein present in the granules of neutrophils and eosinophils. The protein consists

of an N-terminal domain, a proline-rich linker region and a C-terminal domain. The C- and N-terminal domains are each composed of a barrel formed of a short α-helix, a five stranded anti-parallel β-sheet and a long helix. A central β-sheet forms an interface between the two domains[3]. Most research on BPI indicates that the functional domain of the protein lies primarily within the N-terminal domain. Until recently, BPI was best known to function in the defense against bacteria. BPI is bactericidal against mostly Gram-negative bacteria. This process is mediated by outer membrane permeability of bacteria to small hydrophobic substances and increased polymorphonuclear cell phagocytosis and killing. In addition, BPI binds and neutralizes the bacterial endotoxin lipopolysaccharide (LPS) and inhibits the pyrogenic effect of LPS in animals. LPS, the toxic component triggering septic shock, stimulates an inflammatory response via the release of inflammatory mediators such as tumor necrosis factor (TNF) from host inflammatory cells (monocytes)[4]. BPI functions by binding to the lipid A moiety of LPS, which consists of a phosphorylated diglucosamine attached to two fatty acyl chains. BPI is cationic and therefore can bind heparin. This feature of BPI is shared with many blood proteins and growth factors among which are platelet factor 4 (PF4) and fibroblast growth factors. BPI can neutralize the anti-coagulant properties of heparin, which may be a therapeutic tool in patients that are treated with heparin after surgery. Given the shared biological functions with a number of other vasculature regulatory molecules and the chemical similarities that are apparent from the 3-dimensional structure of BPI, we postulated that BPI may share a regulatory role in angiogenesis as a novel endogenous inhibitor.

When human endothelial cells, either from bovine or human macro-vascular (umbilical vein) or from micro-vascular origin (skin), are cultured on tissue culture plastic coated with fibronectin in culture medium containing human serum, addition of BPI in the culture leads to inhibition of growth. This can be measured by cell counting, by measurement of DNA replication after supply of tritiated thymidine for several hours into the culture, or by dye exclusion measurement. Trypan blue exclusion measurements showed that BPI killed bovine endothelial cells and human macrovascular as well as microvascular endothelial cells (Table 6.1). The inhibition of growth by BPI is dose dependent and inhibition was found to be as much as 80% at concentrations of approximately 2 μM. This effect is endothelial cell specific since cultures of other cell types, e.g. fibroblasts and tumor cell lines, appear unaffected. Figure 6.1 shows the appearance of HUVEC and fibroblasts after 3 days of culture under normal conditions or in the presence of 1.8 μM BPI. The most important observation is that endothelial cells appear to die from apoptosis. To investigate this, DNA profiles were analyzed by staining endothelial cells with propidium iodide after DNA extraction. The amount of apoptotic cells was measured by enumeration of subdiploid cells (A_0 peak) on a flow cytometer. A_0 peak measurements indicate that BPI induces apoptosis in endothelial cells. In Figure 6.2, flow cytometric analysis of A_0 peak measurements are visualized for cells treated with BPI or platelet factor 4. As a positive control, endothelial cells were cultured under starvation conditions in culture medium low (1%) in human serum. BPI induced apoptosis in 20% of the cells at a concentration

of 1.8 μM. The induction of apoptosis by BPI was confirmed by cell morphology and TUNEL-staining. In addition, culturing of cells in the presence of the caspase inhibitor ZVAD.fmk, lead to a complete block of the appearance of subdiploid cells. It was questioned whether the detachment of endothelial cells from the matrix was induced by the apoptotic cell death or whether the detachment of cells, called anoikis, induced the apoptosis. The use of the caspase inhibitor z-VAD.fmk undoubtedly demonstrated the latter. In the presence of the inhibitor, no apoptosis occurred, and the cells remained viable, yet still detached from their fibronectin matrix[5].The latter experiments raised the question as to whether the mechanism of BPI is via a blockade of adhesion receptors on endothelial cells. To functionally test this hypothesis, endothelial cell adhesion and migration assays were performed. Wound assays were performed in which HUVEC were cultured in a monolayer on a fibronectin coat. After wounding the monolayer by scraping off cells approximately 40 μm wide using a blunt glass bar, the wound width was measured over time. While control wounds readily grew to confluency, BPI only marginally inhibited this process (Figure 6.3), whereas anginex and PF4 did inhibit markedly the process.

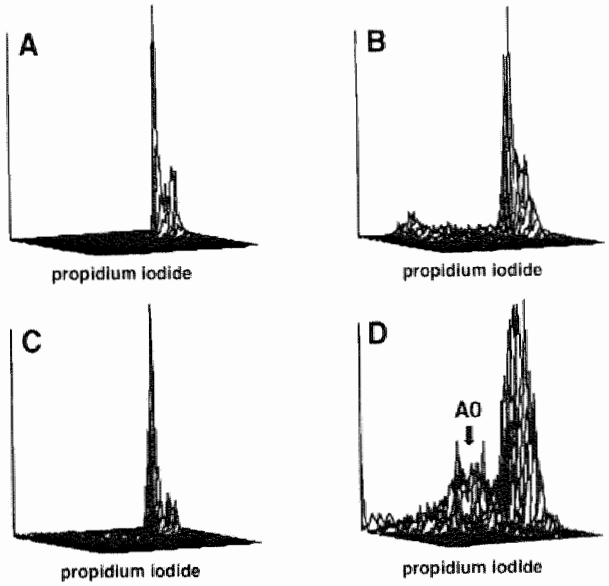

Figure 6.2 *BPI induces apoptosis in endothelial cells.*
Apoptosis was measured by FACS-analysis of subdiploid cells after DNA extraction and propidium iodide staining. Data are presenterd as 3D plots of FL2 (propidium iodide) against forward scatter on the horizontal axes versus the number of cells on the vertical axis. In control cells (A) only 4.4% of the cells is in the subdiploid DNA peak, while when cells are cultured with BPI (B) for 3 days 20.3% of the cells are in the subdiploid peak. Cells cultured with PF4 (C) had a percentage of apoptotic cells comparable to control. Starvation of cells in medium with low serum (1%) (D) was the positive control for detection of apoptotic cells, which was 34.7%.

The effect of BPI on angiogenesis was tested in an *in vitro* angiogenesis assay, which is based on the migration or sprout formation of endothelial cells through a collagen gel. In this assay, bovine microvascular endothelial cells (BCE) were cultured on gelatin-coated beads that were subsequently embedded in a thick collagen type I gel. Sprout formation into the matrix was induced by adding 20 ng/ml bFGF in the medium on top of the collagen gel (Figure 6.4 A). When BPI was added at 1.8 μM to the medium sprout-formation was diminished by 81% (Figure 6.4 B)[5].

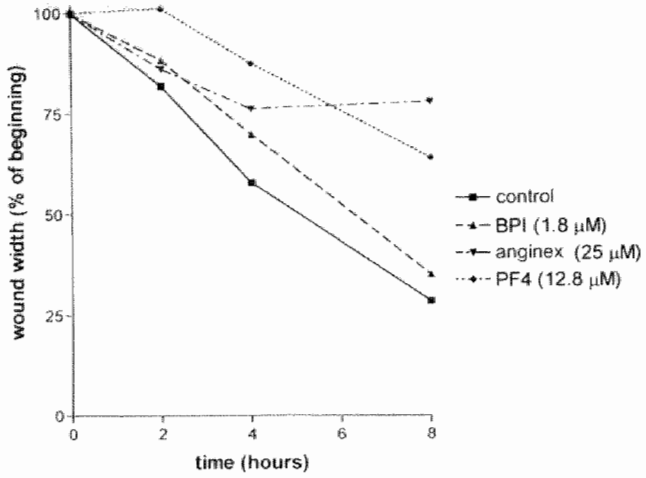

Figure 6.3 *BPI inhibits migration of endothelial cells.*
In a wound assay HUVEC were grown until confluence on a fibronectin coat. A wound was made by scraping of cells, which was measured in time under control condition or when either BPI, anginex or PF4 were added in a concentration of respectively 1.8 μM, 25 μM and 12.8 μM.

To assess effects on *in vivo* angiogenesis, a large array of different assays and models are available (reviewed in[6]). BPI was tested in the chick embryo chorioallantoic membrane, which measures developmental angiogenesis. In fertilized chick eggs, a window was made after 3 days of incubation at 37°C. At day 7, a silicon ring was placed on the chorioallantoic membrane and on day 7, BPI was administered daily on top of the chorioallantoic membrane inside the ring. On day 10, membranes were photographed, and computer analysis showed an inhibition of 42% at 540 nM/l BPI (Figure 6.4 D) as compared to control (Figure 6.4 C)[5].

Beside binding to LPS, BPI is also a heparin binding protein. Heparin binding to proteins can either modulate their function by being a co-factor or block their function. When heparin is added to cultured endothelial cells in the presence of BPI, the effects of BPI are completely abolished[5].

In order to know if BPI inhibits tumor-induced angiogenesis or other types of pathological angiogenesis and can be used as a therapeutic anti-angiogenic agent, further research in tumor and arthritis models is necessary. The development of

BPI as a therapeutic agent in other areas of medical science (infection/septic shock) and the availability of phase I clinical studies will be a clear benefit for developing BPI as an anti-angiogenic agent.

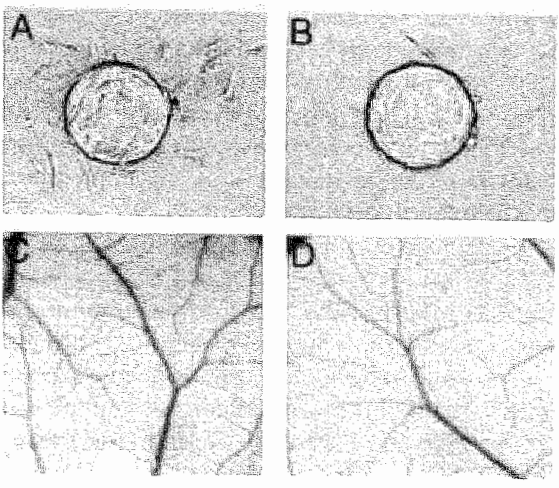

Figure 6.4 *BPI inhibits in vitro and in vivo angiogenesis.*
In an *in vitro* angiogenesis assay bFGF induces sprouting of bovine endothelial cells in a collagen matrix (A). BPI inhibits the sprout formation (B). In a normal CAM (C) the small vessels are being formed on day 10, when BPI is added on top of the CAM small vessel formation is markedly inhibited(D).

BPI belongs to a family of agents that share angiostatic and bactericidal activities

Several molecules known in the literature as being antibacterial, have been reported to inhibit angiogenesis and vice versa. One of the ways to classify antibacterial agents is by their target sites. Four different classes can be distinguished: (i) agents that affect cell wall synthesis, such as beta-lactams, glycopeptides and cycloserine, (ii) agents that inhibit protein synthesis such as the aminoglycosides, neomycin, streptomycin and gentamicin or (iii) nucleic acid synthesis such as sulphonamides, quinolones and rifampicin, and (iv) agents that influence cell membrane function such as polymixins. BPI can be included in the fourth group, since it disrupts membrane permeability and binds LPS from the bacterial membrane. A number of other molecules with both anti-bacterial and anti-angiogenic properties are mentioned below.

One of the first compounds identified to exhibit inhibitory effects on cell growth with specificity for endothelial cells was an analogue of the fungus derived antibiotic fumagillin, O-chloroacetylcarbamoyl fumagillol or AGM-1470/TNP-470[7;8]. This compound was accidentally identified following the observation that in a contaminated culture of bovine capillary endothelial cells, the fungus induced a

gradient of endothelial cell rounding rather than causing cytotoxicity. The mechanism of action of this compound was later found to be the blocking of endothelial cells from entering G_1 phase of the cell cycle, resulting in a decrease in proliferation (reviewed in[9]).

Minocycline is a semisynthetic antibiotic derivative of tetracycline that is clinically available. This compound, found to be anti-angiogenic in the early 1990s[10], is not directly cytotoxic to endothelial cells, but is rather antiproliferative via inhibition of collagenase and thereby halts matrix degradation and subsequent angiogenesis. Interestingly, this effect was found to be endothelial cell specific because astrocytes and pericytes were relatively unaffected in their proliferative response.

Neomycin, an aminoglycoside antibiotic was described to specifically inhibit angiogenesis induced by angiogenins[11]. In this case, inhibition of angiogenesis is mediated by the blockade of nuclear translocation of angiogenin in endothelial cells. This effect is mediated by the inhibition of phospholipase C, an important regulator in the cell cycle. Interestingly, the reported effects are neomycin-specific because other aminoglycoside antibiotics, such as gentamicine, streptomycin, kanamycin, amikacin and paramomycin, are not inhibitory for angiogenesis.

C1027 is an antibiotic, originally described to display potent anti-tumor and metastasis formation effects based on its ability to cleave DNA. This antibiotic, described to be anti-angiogenic, functions as such by interfering with bFGF binding to its receptor[12].

Squalamine is a cationic steroid isolated from the stomach of the dogfish shark *Squalus acanthias*. While the structure and antibiotic activities of this steroid were studied in 1993[13], its anti-angiogenic activity was described in 1998[14]. The latter effect is mediated by inhibition of mitogen-induced proliferation of endothelial cells. Squalamine is effective at inhibiting the establishment of certain xenograft tumors in nude mice and when combined with cisplatin or carboplatin, squalamine dramatically delayed tumor growth in various animal models. The list of antibiotic inhibitors of angiogenesis can be extended, for example, with eponemycin[15], 5-deoxyspergualin[16], Clarithomycin[17], Doxycycline[10], Borrelidin[18], Roxithromycin[19].

Platelet factor 4 (PF4) is one of the first CXC-chemokines to be discovered. It belongs to a large family of structurally related molecules that have a wide variety of different functions. One of these functions is the regulation of vasculature formation. The chemokines that display stimulation of vessel growth, such as IL-8, have an ELR functional motif in the N-terminal region. Other chemokines, such as PF4, Mig and IP-10, lack this motif and are described as negative regulators of angiogenesis (reviewed in[20]). The way in which PF4 inhibits angiogenesis is still uncertain. Several studies with contradictory results have been published. It was reported that PF4 interacts with bFGF and thereby inhibits angiogenesis, whereas some have observed that PF4 inhibits angiogenesis by binding to heparin, and others state that PF4 binds to CXC chemokine receptors present on the surface of endothelial cells. The direct effect of PF4 on endothelial cells was reported to be a cell-cycle arrest leading to synchronization in S-phase. What can be stated with certainty is that PF4 works in a different manner from BPI because, unlike BPI, it

does not induce apoptosis in endothelial cells. PF4 also has been reported to have anti-bacterial properties[21].

Anginex (βpep-25) is a *de novo* designed cytokine-like peptide[22] that inhibits angiogenesis by apoptosis induction in angiogenically-activated endothelial cells and attenuates tumor growth *in vivo*. This peptide was initially designed to form stable β-sheets in aqueous solution and later recognized to have interesting biological properties. Anginex inhibits angiogenesis in a way that appears be similar to BPI, i.e. both induce apoptosis in endothelial cells. Anginex is also capable of killing Gram negative, as well as Gram-positive, bacteria and can neutralize LPS endotoxin.

Recently, an RGD-based peptide was designed to home in on angiogenically activated endothelial cells and induce apoptosis. For this, cyclic RGD (or NGR) peptides were used as the targeting domain and the peptide $_d(KLAKLAK)_2$ was used as the apoptotis-inducing domain, with a chemical linker to connect the peptides. RGD homes in on various integrins, particularly $\alpha_v\beta_3$, which is highly expressed on angiogenically activated endothelial cells. The peptide $_d(KLAKLAK)_2$ is apoptotic, supposedly functioning by disrupting the mitochondrial membrane. The NGR and RGD peptides linked to $_d(KLAKLAK)_2$ were recently shown to inhibit tumor growth in a mouse model with human breast carcinoma xenografts[23]. $_d(KLAKLAK)_2$ has also been demonstrated to be antibacterial[24].

The research on BPI raised the question of why an anti-bacterial protein has angiostatic properties. Other research on endogenous proteins demonstrated that BPI was not the only molecule with these characteristics. This suggested that there may be an evolutionary advantage to combining these properties into one molecule. Inflammation is common to both angiogenesis and bacterial infection[1;25]. Whether the process of angiogenesis is inhibited or stimulated may be dependent on the concentration of the compound. For BPI, for example, endotoxin neutralization occurs at lower concentrations than do antibacterial and angiostatic effects. The concentration at which BPI neutralizes LPS is 10^{-8} M[26], while anti-angiogenic effects are achieved at much higher concentrations of approximately $2x10^{-6}$ M. The need for relatively high concentrations for anti-angiogenic effects, is also the case for PF4, angiostatin, endostatin and anginex. In a phase I clinical study with continuous infusion of BPI in patients with meningococcal sepsis[27], circulating serum levels of BPI were approximately $2x10^{-9}$ M (endogenous levels $2x10^{-11}$ - $5x10^{-10}$ M), which was found to be effective against the infection. This concentration might not be efficacious as anti-angiogenic treatment, however, angiogenesis is a local process and BPI can possibly be present locally in much higher concentrations.

For most angiogenesis inhibitors, the exact mechanism of action is not known. It is tempting to speculate that the angiostatic effects of inhibitors with this dual function, are based on the same principle. Resemblance between endothelial cells and bacteria might seem far fetched; however, there is an interesting idea that should be addressed, namely, mitochondria and bacteria are evolutionarily linked. Angiogenesis inhibitors might affect the mitochondrial and bacterial membranes in

a similar fashion. This has been demonstrated to be the case for $_d$(KLAKLAK)$_2$, which induces apoptosis in endothelial cells by disrupting the mitochondrial membrane, whereby cytochrome C, which is essential in the apoptotic pathway, is released into the cytoplasm. Possible uptake of these mostly cationic anti-angiogenic proteins by receptors on endothelial cells, might be mediated by the negatively charged surface components, such as the glycocalyx of the endothelium.

More bactericidal proteins may soon be discovered to have anti-angiogenic activities, and this might help unravel the mechanistic mystery. The clinical relevance of the anti-angiogenic activity of BPI has yet to be demonstrated, as does the relevance for anti-angiogenic, and possibly antibacterial, activities of other agents described above. The most probable way to do so is by the use of *in vivo* models in which pathological angiogenic diseases can be studied.

Conclusions

Bactericidal/permeability-increasing protein has a dual function in inhibition of angiogenesis as well as bactericidal activity. For the endogenous agents PF4 and BPI, and the synthetically made agents anginex/βpep-25 and $_d$(KLAKLAK)$_2$ similar results have been found. The actual mechanism for most of the angiogenesis inhibitors described in this paper is still uncertain and whether the proteins and peptides can be used in the treatment of cancer or other angiogenesis dependent diseases still has to be shown.

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Chapter 7

General discussion and conclusions

General discussion

The aim of the work presented in this thesis was the development of novel potent angiogenesis inhibitors. Two approaches were used to identify novel angiogenesis inhibitors. The first was a traditional approach. Based on the similarity in structure and biological function of platelet factor 4 (PF4)[1] and bactericidal/permeability-increasing protein (BPI). The angiostatic activity of BPI was investigated. The second was a new approach in which *de novo* peptide design was used based on structure and sequence features of known endogenous β-sheet containing molecules (angiogenesis inhibitors or α-chemokines). The result from this approach was the development of anginex or βpep-25.

The anti-angiogenic protein identified by the traditional approach was BPI. Angiogenesis inhibition is a new function that has been discovered for this endogenous protein. BPI was known as a potent naturally occurring antibacterial[2] and LPS neutralizing agent[3], and is in clinical development for the treatment of sepsis[4]. BPI was found to inhibit angiogenesis both *in vitro* and *in vivo* (in the chorioallantoic membrane of the chicken embryo) at low nM concentrations. Mechanistic studies indicated that BPI inhibited proliferation and adhesion of endothelial cells and the EC detachment lead to the induction of apoptosis. The concentration that inhibited angiogenesis is close to the concentration at which BPI displays LPS neutralizing activity. Whether there is a connection between the antibacterial activity or LPS neutralizing activity and the anti-angiogenic activity of BPI is uncertain. It has been suggested that mitochondria have bacteria as an evolutionary ancestor[5]. It is possible that BPI after binding to the endothelial cell surface membrane is internalized and is transported to the mitochondria. We postulate that it might then interfere with the mitochondrial membrane in the same way as it does with bacterial membranes. Damage to the mitochondrial membrane would then cause the release of cytochrome C activating the caspase cascade ultimately resulting in apoptosis.

It has been suggested that the anti-angiogenic activities of PF4 might play a role in limiting (excessive) angiogenesis during wound healing. After closing a wound platelets would release PF4 stopping further new vessel formation. Similarly BPI might be released from neutrophils during an anti-inflammatory response thus restoring the angiogenic balance once inflammation begins to be down regulated.

The receptor for BPI on endothelial cells has not yet been identified. Heparin was, however, able to block the apoptotic cascade induced by BPI. Heparin is known to bind to BPI and it is possible that it blocks its activity by preventing receptor binding this way. However, it also is possible that it binds to the receptor and thereby blocks the activity of BPI. The receptor thus might well be a heparin binding moiety.

Whether BPI can be used to inhibit angiogenesis in cancer patients remains to be determined. If it transpires that plasma concentrations that have been achieved in patients with sepsis are in the same range as those that inhibit angiogenesis *in vitro* then the clinical development of BPI as an angiogenesis inhibitor can proceed rapidly.

Anginex was developed by *de novo* peptide design. It was selected from a library of 33-mers, that all had the β-sheet folding capacity. This library was designed to make stable, water soluble and strongly effective angiogenesis inhibitors, which were based mainly on the structure and active sequence of platelet factor 4 (PF4)[1] and BPI[6;7]. The results found during the investigation of BPI and the published results on the anti-angiogenic activity of PF4 formed the basis for studying the anti-angiogenic activity of anginex. Anti-angiogenic activity was tested in several assays, both *in vitro* and *in vivo*.

Two different *in vitro* angiogenesis assays were used that measure the complete angiogenesis process including cell growth and migration. The first was an adaptation of an earlier described assay system[8]. In this assay endothelial cells are grown to a monolayer and induced to form sprouts in a collagen gel under stimulation by bFGF. The sprout formation in this assay was, however, highly variable and inconsistently present. The assay was improved by culturing the endothelial cells on gelatin coated beads that are then embedded inside the matrix[9]. The sprouts that are formed from one bead are easily quantifiable. Anginex inhibited angiogenesis in this assay by 80%. It would be preferable if this assay could be performed not solely in a collagen gel, but also on other extracellular matrix components. This was, however, not possible because of technical difficulties with the other matrices and the high cost of matrigel, which makes it unpractical for use as a high throughput angiogenesis assay.

The first *in vivo* model used was the chorioallantoic membrane (CAM) assay in fertilized chicken eggs[10]. This is a model commonly used in angiogenesis research and measures developmental angiogenesis. It is an *in vivo* assay which is easy to use for several reasons. Firstly, the formation of new blood vessels can be followed over time. Secondly, the vessels are easy to quantify by a method developed previously[11]. Thirdly, drugs can be applied to the membrane. Fourthly, the chicken embryos are not included in experimental animal legislation. We applied anginex to the membrane and measured developmental angiogenesis and could see a concentration dependent decrease in vessel density. Others have developed a cancer model in the CAM[12] that measured tumor fragment induced angiogenesis. It is also possible to investigate angiogenesis induced by a growth factor in the CAM. These last two methods could have been used in future research, however we preferred to assess the *in vivo* anti-angiogenic activity of anginex in other models that measured either growth factor or tumor induced angiogenesis. A second *in vivo* angiogenesis model used was the matrigel plug assay[13]. In this assay matrigel is injected on the flank of a mouse. There are different ways to quantify the vessel

formation. Some investigators measure hemoglobin, but we chose to stain sections with CD31 antibody and determined vessel density. One of the flaws of the assay is that the new capillary formation cannot be followed over time. An advantage of the assay is, however, that angiogenesis can be induced by only one growth factor, and that inhibition of this specifically induced angiogenesis can be effected either by mixing the test compound into the matrigel plug or by the systemic treatment of the animal. In this assay, anginex inhibited the bFGF-induced angiogenesis by mixing it in the matrix. Others also use this assay to investigate tumor cell induced angiogenesis. The extra value of this approach compared to assessing complete tumor induced angiogenesis is questionable.

Since the research presented in this thesis lies in the field of oncology, the anti-tumor efficacy of anginex was investigated in mice. Two models were used. The first model was the Ls174T colon carcinoma model[14]. In this model immune incompetent mice were inoculated with the human colon carcinoma cell line Ls174T. In a tumor growth prevention assay treatment with anginex was started on the same day by loco-regional treatment. The loco-regional treatment was chosen because of the small size of anginex (3.8 kD), which suggested that it might be excreted rapidly making it difficult to achieve sustained concentrations of peptide over prolonged periods of time. A negative side effect that occurred was the formation of a precipitate of the peptide at the site of injection. This made it difficult to accurately measure tumor size. The formation of the precipitate and the clear inhibition of tumor growth indicated that low concentrations of peptide would be sufficient to produce tumor growth inhibition. This and the results from studies done in Minneapolis lead to the development of the second model. In this model anginex was continuously delivered via an osmotic mini-pump that was placed subcutaneously in the flank of immune-competent C57BL/6 mice that were inoculated with the rapidly growing and metastasis forming melanoma cell line B16F10[15]. Anginex was administered at three different doses that were all lower than those used in the first model used. A concentration dependent inhibition of tumor growth was observed with a maximal inhibition of 63% on day 16 when treated with 0.4 mg/animal/day (14 mg/kg/day). This inhibitory effect was, however, only 5% more than was seen with 0.2 mg/animal/day (7 mg/kg/day). The B16F10 melanoma cell line is a very aggressive tumor, which is frequently used in angiogenesis research. The concentrations used in this model were comparable to the concentrations (in mg/kg) of other angiogenesis inhibitors used *in vivo* in the mouse models described in literature. Examples of such compounds include canstatin[16], suramin[17] and endostatin[18]. The use of the mini-osmotic pump is an improvement compared to repeated s.c. injections, because the continuous treatment meant that anti-tumor effects were observed at lower doses of peptide. No toxic effects were observed with anginex in both mouse models indicating that toxicity should not be problem in the human situation. Treatment with TNP-470 however induced a weight decrease, indicating that this compound may produce toxicity in humans (data not shown).

Mechanistic studies were performed to gain a greater insight into the mechanism by which anginex inhibits angiogenesis. When anginex was added to endothelial cells in culture it visibly induced detachment of endothelial cells from the underlying matrix. The influence of anginex on cell adhesion was, therefore, subsequently tested in an adhesion assay. Anginex did not directly block adhesion, but when adherent cells were incubated with anginex for 24 hours detachment from several matrix molecules was observed. This was seen with fibronectin, vitronectin and hyalorunic acid and indicated a mechanism of action involving down-regulation of adhesion molecule expression. This hypothesis was tested by flow cytometric analysis of endothelial cells cultured on a fibronectin matrix with anginex and subsequently stained with antibodies directed to adhesion molecules that are involved in matrix binding. A down-regulation of all adhesion molecules tested, $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_2\beta_1$ and CD44 was observed. Adhesion of endothelial cells to the extracellular matrix is important for two reasons. Firstly endothelial need to adhere to the matrix molecules in order to migrate. Secondly endothelial cells need to adhere in order to be protected from anoikis (apoptosis induced when cells become detached from the cell matrix due to removal of survival signals transmitted via the integrins mentioned above).

Modulation of endothelial cell migration by anginex was tested in a migration wound assay. In this assay a confluent monolayer of endothelial cells is wounded and culture medium is replaced by medium with the test substances[9]. The endothelial cells in the control condition were able to close the wound, however when anginex was added to the wounded monolayer the endothelial cells were unable to close the wound, indicating a decreased ability of the endothelial cells to migrate. The wound assay was performed with human as well as mouse endothelial cells and similar results were obtained. Next to migration, angiogenesis also depends on endothelial cell growth, which is a consequence of a balance between proliferation and cell death. Proliferation of endothelial cells was measured with the [^{3}H]-thymidine incorporation assay, which is probably still the most accurate method available. Inhibition of proliferation by anginex was endothelial cell specific, but not species specific since both human, bovine, rat and mouse endothelial cells were all sensitive to anginex treatment. Our hypothesis is that exposure to anginex leads to down regulation of integrins resulting in detachment of cells, which then induces apoptosis (anoikis). The detachment and apoptosis induction by anginex was not seen when cells were cultured on a matrix of collagen type I. Collagen coating has been shown to protect endothelial cells from apoptosis[19]. This might be explained by the fact that binding to collagen gives a stronger survival signal to the cells than binding to other matrix components, even in spite of slight downregulation of $\alpha_2\beta_1$ integrin expression if this even occurs when cells are cultured on collagen.

Apoptosis induction was measured in several ways. The first was by determination of the percentage of subdiploid DNA containing cells in the flow cytometer after DNA extraction and propidium iodide staining of the cells. Apoptosis induction was confirmed by examining the morphological features of the cells, TUNEL staining of the cells, culture of the cells in the presence of the

caspase inhibitor ZVAD.fmk[20] and measurement of caspase 3 activation[21]. All these methods confirmed that anginex induced apoptosis in endothelial cells. This apoptosis induction was also seen in endothelial cells cultured with BPI, but not in cells cultured with PF4. This indicates that PF4 is a cytostatic molecule, whereas BPI and anginex are cytotoxic. For anginex the active residues have been investigated and were found to be L5, V7, K10, K17, I20, V22, L24. PF4 also contains K10 and L24, but BPI contains both these amino acids and K17. It is possible that the K17 moiety is responsible for these differences between BPI and PF4. The influence on cell cycle regulating proteins was measured for anginex, because perturbation of these proteins has been reported for PF4. Anginex showed the same changes in p53, p21 and pRb expression as has been found for PF4[22], indicating inhibition of the cell cycle. In the presence of anginex there was, however, not only a cell cycle arrest but also induction of apoptosis. A clear explanation for this observation is not yet available. Most probably anginex inhibits angiogenesis in the same fashion as does BPI, but this has not yet been fully investigated.

It was shown (by histochemical en electron microscopic analysis) that anginex binds to the membrane of endothelial cells and that after internalization it becomes localized in cytoplasmic vesicles. The receptor present on endothelial cells and which is probably the first step leading to the anti-angiogenic effects of anginex has not yet been identified, but will most probably be discovered in the near future. Although receptor binding and internalization seems the most likely explanation of the cellular uptake and induction of the biological activities of anginex based on the available data we cannot currently completely exclude the fact that cell uptake may be occurring via pinocytosis

The mechanism of apoptosis induction in cells can be roughly divided into two pathways. In one pathway receptor binding leads directly to caspase activation, which ultimately leads to apoptosis. In the other pathway stress factors (e.g. serum starvation or DNA damage) causes the release of cytochrome C from the mitochondrion[23] leading to caspase activation. Whether the apoptosis induced by anginex results from receptor mediated apoptosis induction or via damage to the mitochondrial cell membrane, causing release of cytochrome C as has been postulated for BPI (see above), cannot be determined from the work conducted for this thesis. Circumstantial evidence for the receptor-mediated route was derived from experiments in which anginex was coupled to large beads, thus preventing internalization. In these experiments apoptosis was still induced in the endothelial cells (data not shown). These preliminary results that although anginex is internalized into the endothelial cells after binding to a membrane receptor the binding to this receptor may have already activated a signaling pathway eventually leading to apoptosis induction.

Data presented in this thesis demonstrate that anginex stops cell cycle progression by reducing phosphorylation of pRb. This reduction in phosphorylation was the consequence of the up-regulation of p53, which initially leads to inhibition of cell cycle progression and if conditions are unfavorable for the cell to apoptosis. The apoptosis induced in this case can be seen as stress

related leading to cytochrome C release from the mitochondria (this was however not measured). It is also known that anginex induces anoikis, which also can be seen as a stress related phenomenon, since loss of binding to the extracellular matrix destabilizes the cytoskeleton and this can lead to apoptosis. This was confirmed by the fact that when cell cycle progression was halted the cells still detached but did not undergo apoptosis. This suggests that anginex interferes with cell cycle regulating molecules in order to induce apoptosis. Another feature of anginex-induced apoptosis that indicates that the stress-induced pathway is the more important is the fact that apoptosis occurs slowly, whereas induction of apoptosis by binding to a receptor that has a death domain is a rapidly occurring process. Whether internalization as such or receptor mediated internalization is an essential prerequisite for apoptosis induction remains to be determined. Other preliminary experiments (not shown) showed that anginex directly bound caspase 3 and lead to its activation. A similar phenomenon has been described for the angiogenesis inhibiting RGD peptide[24]. These complex findings suggest that multiple pathways for inducing apoptosis after exposure to anginex may well be operational. Which are the dominant pathways remains to be determined.

Both anginex and BPI differ from most angiogenesis inhibitors in the way they inhibit angiogenesis. Most angiogenesis inhibitors only act on just one part of the angiogenic cascade. For example they might inhibit one of the following pathways: endothelial cell growth, migration, vessel maturation or MMP activation. Both anginex and BPI inhibit endothelial cell growth by a number of different pathways suggesting that they may be more effective angiogenesis inhibitors than molecules that only work via a single effector mechanism.

The angiogenesis inhibitors in focus in the thesis, anginex, BPI and PF4, not only display angiostatic activity but also have bactericidal activity. Whether there is a commonality in both these activities has not been fully determined. Other angiogenesis inhibitors such as minocyclin[25] and squalamine[26] also possess antibacterial properties. A structure function relationship analysis will probably yield more information as to whether the antibacterial and anti-angiogenic activities are linked. It will also be of importance to determine whether these inhibitors also inhibit angiogenesis induced by inflammation.

Future directions

Angiogenesis inhibition shows great promise as a new cancer treatment. Some 100 angiogenesis cancer trials are listed on the National Cancer Institutes homepage, describing trials with approximately 40 angiogenesis inhibitors. These data do not even include publications in the regular press relating to angiostatin[27] and endostatin[28] (see National Cancer Institute homepage). Many of the angiogenesis inhibitors in clinical trials are being administered in combination or together with chemotherapeutic drugs. The aim of this latter approach is to reduce

tumor load with conventional chemotherapy and then inhibit re-growth of the tumor with the angiogenesis inhibitor. Synergy between these two treatment modalities may be occurring in a more subtle way. For example vascular endothelial growth factor (VEGF) is also known as vascular permeability inducing factor. The high expression of VEGF in tumors causes leakage of proteins into the interstitial space causing a high intra-tumor pressure. This pressure is able compress the immature vasculature leading to a decrease in perfusion of some areas of the tumor. This decreased perfusion would mean that chemotherapeutic agents would penetrate poorly into the tumor reducing their efficacy. Strategies designed to inhibit the biologic activity of VEGF should reduce interstitial pressure producing improved blood flow and better access of the drugs to the tumor cells. Further more there are accumulating data that at least some chemotherapeutic agents may be angiogenesis inhibitors in their own right.

For BPI many studies are underway to determine its role in the treatment of sepsis. Pre-clinical studies have to be done to see if BPI has anti-tumor effects or can be used for the treatment of other pathological disorders that are dependent on excessive angiogenesis. Currently more pre-clinical studies are on going with anginex. These include combination studies with chemotherapeutic agents and other angiogenesis inhibitors. Further the identity of the cell surface receptor and the mechanism of action of anginex are being actively pursued.

Based on research that has been done to examine the amino acids necessary for anti-angiogenic effect mimetics are being made. These mimetics incorporate the residues important for the biological effects of anginex. The aim of this research is to produce small molecules with a favorable pharmacological profile, which are orally bioavailable. The first of these molecules are currently being tested for their anti-angiogenic activity and further improvements will be made in the near future. These mimetics are also more amenable to large scale manufacturing making them attractive to the pharmaceutical industry. It is our hope that the research presented in this thesis will lead to development of novel anti-angiogenesis and anti-bacterial agents which will benefit patients in future years.

Conclusions

BPI is a strong angiogenesis inhibitor that inhibits angiogenesis *in vitro* as well as *in vivo*. Anginex is a *de novo* designed peptide that inhibits angiogenesis by induction of apoptosis, which is probably the result of the induction of anoikis, and inhibition of migration of endothelial cells. The peptide inhibits tumor growth in mice by inhibition of angiogenesis. Anginex and also BPI show potential for further pre-clinical development and their eventual examination in clinical trials. Only such trials will demonstrate whether they will prove to be a useful new tool to combat cancer.

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Chapter 8

Summary/Samenvatting

Summary

The research field of angiogenesis is currently a major focus in cancer research. Angiogenesis is involved in normal physiological processes of tissue development as well as in several pathologic disorders among which are cancer, rheumatoid arthritis, atherosclerosis, diabetic retinopathy and psoriasis. Chapter 1 gives an introduction to the cancer related angiogenesis research field and to the research presented in this thesis. Many researchers are searching for novel angiogenesis inhibitors and many approaches are being used in this search. Most anti-angiogenesis proteins were found after testing angiostatic activity of endogenous molecules. In this thesis, a novel approach was used to develop new angiogenesis inhibitors by using a combination approach employing basic folding principles and incorporating short sequences from the β-sheet domains of vasoactive proteins. This approach was used to generate a library of related β-sheet forming 33-mers that are stable and water soluble, structure of which was based on the 3D-structure of α-chemokines such as platelet factor 4 and interleukin 8, and the β-sheet domain containing molecule bactericidal/permeability-increasing protein (BPI). Testing of this library revealed a number of highly active anti-angiogenic molecules. In initial reseach the parent molecules were tested in angiogenesis assays. The first important achievement of this research was that an anti-angiogenic function has been ascribed to BPI (Chapter 2). BPI, until now, has only been known to function in killing bacteria and neutralizing the effects of bacterial endotoxin lipopolysaccharide. At the sub-μM range, BPI shows a concentration dependent inhibition of endothelial cell (EC) proliferation which is mediated by cell detachment and subsequent induction of apoptosis. As measured by flow cytometric analysis of the percentage of subdiploid cells, apoptosis induction was half-maximal at about 250 nM BPI. Apoptosis was found to be EC specific and was confirmed by quantification of cells with nuclear fragmentation. In an *in vitro* collagen gel-based angiogenesis assay, BPI at 1.8 μM inhibited tube formation by 81% after only 24 hours. Evidence for *in vivo* inhibition of angiogenesis was obtained using the chorioallantoic membrane (CAM) assay in which BPI was seen to be significantly effective at concentrations as low as 180 nM. This newly discovered function of BPI might provide a possible therapeutic modality for the treatment of various pathologic disorders, which are dependent on angiogenesis.

The library of novel β-sheet-forming peptide 33mers, has been screened for anti-proliferative and apoptotis inducing activities. One of these designed peptides (βpep-25) named anginex, was observed to be potently anti-angiogenic, as described in chapter 3. Anginex specifically inhibits vascular endothelial cell proliferation and induces apoptosis in these cells as evidenced by flow cytometric detection of sub-diploid cells, TUNEL-analysis and cell morphology. Anginex also inhibits endothelial cell adhesion to, and migration on, different extracellular matrix components. Inhibition of angiogenesis *in vitro* is demonstrated in the sprout formation assay and *in vivo* in the chick embryo chorioallantoic membrane angiogenesis assay. Comparison of active and inactive βpep-sequences allows

structure-function relationships to be deduced. Five hydrophobic residues appear to be crucial to activity (Chapter 3).

In order to investigate the potential of anginex to be used for cancer treatment, the efficacy was studied in *in vivo* mouse models (Chapter 4). Anginex was tested for anti-tumor effects in the B16F10 melanoma model in C57BL/6 mice, and was found to inhibit tumor growth dose dependently for approximately 60% at 0.2 mg/animal/day when continuously administered using osmotic minipumps. The mechanism of action was studied using the mouse EC lines SVEC4-10 and TME and freshly isolated mouse EC. Anginex inhibited proliferation for 74% in TME and for 50% in SVEC4-10 and induced apoptosis in these cell lines as measured by subdiploid peak analysis. When SVEC4-10 or TME were cultured for 3 days in the presence of 75 μM anginex, 8% and 12% of the cells, respectively, revealed an apoptotic subdiploid DNA profile. Apoptosis induction by anginex was also demonstrated in freshly isolated and activated EC from mouse lung and heart tissues by FACS analysis of DNA fragmentation in CD31 positive cells. To study regulation of migratory activity of EC, the wound assay was used. Significant inhibition of migration could already be observed 4 hours after wounding the monolayer. At 75 μM, anginex potently inhibited up to 90% of migration of both SVEC4-10 and TME cells, when measured after 24 hours. The latter may suggest that specific cell adhesion processes may be regulated by anginex (Chapter 4).

In chapter 5 a detailed description of the anti-angiogenic mechanism of anginex is presented. The specific binding of the peptide to the membrane of EC and its internalisation in time by endocytosis is demonstrated. Anginex induced detachment and subsequent apoptosis in EC. This was demonstrated by the fact that when the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD.fmk) was added to a culture of endothelial cells with anginex, cells still detached but did not undergo apoptosis. The influence of anginex on cell cycle regulating molecules, like p21, p53 and pRb were similar as for PF4, however the apoptosis induction of anginex in EC were similar to that of BPI. Anginex inhibited angiogenesis *in vitro* and inhibits tumor growth *in vivo* in two different mouse models, the Ls174T human colon cancer model and the B16F10 mouse melanoma model. Evidence was presented that the tumor growth inhibition was mediated by inhibition of angiogenesis. The studies presented in chapter 3 through 5 revealed promising results for the development of anginex for clinical use as therapeutic for diseases with excess angiogenic like cancer, diabetic rethinopathy, psoriasis and atherosclerosis.

Chapter 6 reviews the current knowledge on angiogenesis inhibitors and their possible role as anti-bacterial agents, which is exemplified by BPI, which was discovered as a bacterial permeabilizing and LPS neutralizing protein, and later was found to inhibit angiogenesis. In addition, the issue of commonality between antibacterial and antiangiogenic functions is addressed in this chapter.

The research in this thesis describes the novel function of BPI as angiogenesis inhibitor and the development of the new synthetic angiogenesis inhibitor anginex. Future research will address the search for anginex' receptor and signaling pathways, as well as further pre-clinical and clinical testing to develop anginex for novel treatment regimens in a broad array of angiogenic diseases.

Samenvatting

Angiogenese, de vorming van nieuwe bloedvaten uit reeds bestaande bloedvaten, is een proces dat onder verschillende omstandigheden optreedt. Bij de ontwikkeling van weefsels, bijvoorbeeld tijdens de embryogenese en wondheling, speelt angiogenese een belangrijke rol. Ook bij ziekten, zoals kanker, rheumatoide arthritis en cardiovasculaire aandoeningen, is angiogenese een kritieke factor. Met name tumoren zijn voor hun zuurstof en voedselvoorziening afhankelijk van angiogenese. Remming van angiogenese kan daarom als een therapeutische aanpak van tumorgroei worden gezien. Het onderzoek naar het blokkeren van het proces van angiogenese heeft zich ontwikkeld tot een wetenschap en heeft vele onderzoekers aangezet tot de zoektocht naar nieuwe angiogeneseremmers. Deze kunnen zowel van endogene, exogene, of synthetische oorsprong zijn en kunnen worden gebruikt voor de behandeling van patienten. Vele onderzoeksbenaderingen werden gebruikt bij deze zoektocht. De in dit proefschrift beschreven aanpak voor het ontwikkelen van nieuwe angiogenese remmers was het *de novo* design van een serie 33-meer peptiden, waarvan de sequentie en structuur gebaseerd zijn op plaatjes factor 4 (PF4), interleukine 8 (IL8) en 'bactericidal/permeability-increasing protein' (BPI). In initieel onderzoek werden PF4, IL8 en BPI getest in angiogenese assays. Het eerste belangrijke resultaat van dit onderzoek was de identificatie van BPI als angiogeneseremmer (Hoofdstuk 2). BPI is reeds lange tijd bekend als antibacterieel middel en is in staat om lipopolysaccharide (endotoxine) te neutraliseren. BPI is in staat om bij sub-μM concentraties endotheelcelproliferatie te remmen, hetgeen gemedieerd wordt door het losraken van cellen en daaropvolgende apoptose-inductie. Met de flow cytometer werd het percentage subdiploïde cellen bepaald, waaruit bleek dat de apoptose-inductie halfmaximaal was bij een concentratie van 250 nM. De apoptose inductie was endotheelcel specifiek en apoptose werd bevestigd door het microscopisch beoordelen van kernfragmentatie bij cellen. Spruitvorming van endotheelcellen in een collageengel, die geïnduceerd kan worden door toevoeging van fibroblastengroeifactor, werd voor 81% geremd door toevoeging van BPI. Als *in vivo* model voor angiogenese werd de chorioallantoidmembraan van een kippenembryo gebruikt. In dit model was BPI al bij een concentratie van 180 nM in staat om de vorming van nieuwe vaten significant te remmen. Deze nieuw ontdekte functie van BPI biedt mogelijkheden om BPI verder te ontwikkelen tot therapeuticum voor de behandeling van angiogenese afhankelijke ziekten.

Nieuwe β-sheet vormende 33-meer peptiden werden ontworpen door gebruik te maken van een combinatieaanpak. Eén van de aldus ontworpen peptiden, anginex of βpep-25, bleek een sterke anti-angiogene werking te hebben (Hoofdstuk 3). Anginex remt specifiek de proliferatie van endotheelcellen en induceert ook apoptose in deze cellen zoals aangetoond is met flowcytometrische detectie van subdiploïde cellen, TUNEL-analyse en morphologische kenmerken van cellen. Anginex remt bovendien endotheelceladhesie aan en migratie op verschillende extracellulaire matrix componenten. Remming van *in vitro* angiogenese is

aangetoond in de spruitvormings assay en remming van *in vivo* angiogenese in de chorioallantoidmembraan van het kippenembryo. Structuur-functierelaties kunnen bepaald worden aan de hand van het vergelijken van actieve en inactieve sequenties. Vijf hydrofobe aminozuurreciduen bleken essentieel te zijn voor de anti-angiogene activiteit (Hoofdstuk 3).

De effectiviteit van anginex werd getest in *in vivo* muis modellen om de behandelingsmogelijkheden van kanker met het peptide te onderzoeken (Hoofdstuk 4). Anginex werd getest voor anti-tumoreffecten in het B16F10-melanoommodel in C57BL/6 muizen. Tumorgroei werd in dit model met 59% geremd, wanneer de muizen werden behandeld met 0,2 mg/muis/dag bij een continue toediening met osmotisch minipompjes. Mechanistische studies werden gedaan met de muis endotheelcellijnen SVEC4-10 en TME en met vers geïsoleerde muis endotheelcellen. Anginex remde 74% van de proliferatie van TME en 50% van SVEC4-10 en induceerde apoptose in deze cellijnen. Wanneer SVEC4-10 of TME gedurende 3 dagen werden gekweekt met 75 μM anginex was respectievelijk 8% en 12% van de cellen in apoptose. Apoptose inductie door anginex werd ook aangetoond in versgeïsoleerde en geactiveerde endotheelcellen uit hart en longweefsels van muizen met behulp van FACS-analyse van DNA-fragmentatie in CD31-positieve (endotheel)cellen. Om effecten op migratie te onderzoeken werd de wond assay gebruikt. Significante remming van migratie kon reeds 4 uur na het verwonden van de endotheelcelmonolaag worden gezien. Een maximale remming van 90% kon worden waargenomen na incubatie met 75 μM anginex na 24 uur. Dit laatste suggereert dat anginex specifieke celadhesieprocessen reguleert (Hoofdstuk 4).

In hoofdstuk 5 wordt de specifieke binding van het peptide aan de membraan van endotheelcellen en de internalisatie ervan in de tijd beschreven. Ook wordt hier aangetoond dat anginex het loslaten van endotheelcellen van de extracellulaire matrix en de daaropvolgende apoptose induceert. Wanneer de caspase remmer ZVAD-fmk wordt toegevoegd aan de cellen, lieten de cellen nog steeds los, maar gingen niet meer in apoptose. De invloed van anginex op celcyclus-regulatiemoleculen, p53, p21 en pRB is hetzelfde als van plaatjes factor 4, echter, anginex induceert ook nog apoptose, terwijl PF4 geen apoptose induceert. Anginex remt angiogenese en tumorgroei *in vivo* in twee verschillende muismodellen, Ls174T-coloncarcinoom en B16F10-melanoom (Hoofdstuk 5). De studies gepresenteerd in hoofdstuk 3 tot en met 5 tonen veelbelovende resultaten voor de verdere ontwikkeling van anginex voor gebruik in de kliniek. Hoofdstuk 6 beschrijft de huidige kennis over angiogeneseremmers en hun moglelijke anti-bacteriele werking, zoals dat voor BPI beschreven is.

Het onderzoek beschreven in dit proefschrift heeft geleid tot de identificatie van BPI als angiogeneseremmer en de ontwikkeling van een nieuwontworpen angiogeneseremmer anginex. Anginex remt de tumorgroei middels angiostatische activiteit. Mechanistische studies hebben aangetoond dat anginex angiogenese remt door apoptose in endotheelcellen te induceren en migratie van deze cellen te remmen. Er is goede hoop dat anginex verder ontwikkeld kan worden tot therapeutisch middel voor de behandeling van kanker.

Curriculum vitae

Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 25 mei 1976 in Geleen. In 1988 begon zij haar VWO-opleiding aan het Bisschoppelijk College te Sittard waarvan zij in 1994 het diploma behaalde. In datzelfde jaar begon ze met de studie Gezondheidswetenschappen aan de Universiteit Maastricht, met als afstudeerrichting Biologische Gezondheidkunde. Op 31 oktober 1998 werd het doctoraal examen behaald. Tijdens de studie werden twee stages afgerond. De eerste vond plaats binnen de afdeling Interne Geneeskunde van het Academisch Ziekenhuis te Maastricht en de tweede stage vond plaats binnen de afdeling Pathologie van hetzelfde ziekenhuis. Vanaf 1 november 1998 was de schrijfster in dienst als AIO bij de afdeling Interne Geneeskunde. Het onderzoek dat tijdens deze periode gedaan is heeft geleid tot de bewerking van dit proefschrift.

List of publications

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List of publications

Relou IA, Damen CA, van der Schaft DWJ, Groenewegen G, Griffioen AW: Effect of culture conditions on endothelial cell growth and responsiveness. Tissue Cell 30: 525-530, 1998

Van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW: Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. Blood 96: 176-181, 2000

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Mayo KH, Flader C, Dings RPM, Hargittai B, van der Schaft DWJ, van Eijk LI, Haseman J, Hoye TR, Griffioen AW: Designing a mimetic of angiostatic anginex. Submitted

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Van der Schaft DWJ, Schultz H, van Eijk LI, Griffioen AW: The influence of BPI ANCAs on the anti-angiogenic activity of BPI. In preparation

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Dankwoord

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